Ranaviruses:
Detection, differentiation and host immune response

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Abstract

Ranaviruses cause systemic infection in fish, amphibians and reptiles. Disease outbreaks associated with ranaviruses have been reported worldwide, and their harmful impact on aquaculture and natural populations of host animals has drawn attention to this group of viruses. In this research, genetic relationships among members of genus *Ranavirus*, including several known and less studied isolates, were investigated. Sequences of several viral genes, including major capsid protein (MCP), DNA polymerase (DNApol), neurofilament triplet H1-like protein (NF-H1), ribonuclease reductase α and β subunits (RNR-α and -β) and RNase III, were obtained and the phylogenetic analyses based on the sequence data were performed. The results confirm that the genus *Ranavirus* encompasses genetically divergent isolates. Most of the ranaviruses characterized to date are closely related to epizootic haematopoietic necrosis virus (EHNV) and frog virus 3 (FV3). Santee-Cooper ranaviruses and grouper iridoviruses cluster separately from the main group of ranaviruses.

Methods based on PCR and subsequent restriction enzyme analysis (REA) of viral DNApol and NF-H1 genes were developed for the detection and differentiation of ranaviruses. DNApol PCR was able to detect 13 different ranavirus isolates. The most divergent isolates were distinguished with REA of the DNApol gene, whereas differentiation of the more closely related ranaviruses was accomplished with REA of the NF-H1 gene. Quantitative PCR (qPCR) based on the viral DNApol gene was developed in order to detect a wide range of ranaviruses and to estimate the number of virions by using a standard curve. Another qPCR method targeting the fish glucokinase (GK) gene was developed for the quantitation of piscine host cells. Using both DNApol and GK qPCR, it was possible to obtain a viral load value, virions per host cell, from ranavirus-infected cell cultures and fish tissues.

Pathogenicity of nine different ranavirus isolates to rainbow trout (*Oncorhynchus mykiss*) was studied. None of the isolates caused signs of disease, but virus was isolated from few of the fish collected during the challenge trial. The results indicate that persistent ranavirus infections may occur in certain conditions and that rainbow trout could act as a vector species for ranaviruses. In addition, fish epithelial cells were infected with four different ranaviruses in order to study the host immune response to ranavirus infection. The mRNA expression of five host immune response genes was measured with qPCR at seven time points after viral inoculation. Based on the results, even though all ranavirus isolates induced apoptotic changes in the epithelial cells, the immune response elicited varied between different ranaviruses. A strong pro-inflammatory response was elicited by FV3 and EHNV, whereas European catfish virus and doctor fish virus induced a mild increase in the expression of regulatory cytokine TGF-β. Gene expression of beta-2 microglobulin was enhanced by all viral isolates, indicating that a pathogen presentation pathway based on MHC class I molecules was activated due to ranavirus infection.

Contents

List of original publications

This thesis is based on the following publications:

I Holopainen R, Ohlemeyer S, Schütze H, Bergmann SM, Tapiovaara H (2009) Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. Diseases of Aquatic Organisms 85: 81-91. doi:10.3354/dao02074. www.int-res.com/articles/dao_oa/d085p081.pdf

II Ariel E, Holopainen R, Olesen NJ, Tapiovaara H (2010) Comparative study of ranavirus isolates from cod (*Gadus morhua*) and turbot (*Psetta maxima*) with reference to other ranaviruses. Archives of Virology 155: 1261-1271. doi:10.1007/s00705-010-0715-z.

III Holopainen R, Honkanen J, Bang Jensen B, Ariel E, Tapiovaara H (2011) Quantitation of ranaviruses in cell culture and tissue samples. Journal of Virological Methods 171: 225-233. doi:10.1016/j.jviromet.2010.11.004.

IV Holopainen R, Tapiovaara H, Honkanen J. Expression analysis of immune response genes in fish epithelial cells following ranavirus infection (2012). Fish and Shellfish Immulogy 32: 1095-1105. doi: 10.1016/j.fsi.2012.03.011.

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Abbreviations

ATV	Ambystoma tigrinum virus
BF-2	bluegill fry (cell line)
BIV	Bohle iridovirus
β2M	beta-2 microglobulin
CPE	cytopathic effect
Ct	cycle threshold
CTL	cytotoxic T lymphocyte
DFV	doctor fish virus
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
DNApol	DNA polymerase
ECV	European catfish virus
EHNV	epizootic haematopoietic necrosis virus
ELISA	enzyme-linked immunosorbent assay
EPC	epithelioma papulosum cyprini (cell line)
ESV	European sheatfih virus
FHM	fathead minnow (cell line)
FV3	frog virus 3
gDNA	genomic DNA
GIV	grouper iridovirus
GK	glucokinase
GV6	guppy virus 6
HO	Hoechst (stain)
ICTV	International Committee on the Taxonomy of Viruses
IFAT	indirect fluorescent antibody test
IL-1β	interleukin 1 beta
IL-10	interleukin 10
IP	intraperitoneal
ISKNV	infectious spleen and kidney necrosis virus
KHV	koi herpesvirus
LCDV	lymphocystis disease virus
LMBV	largemouth bass iridovirus
MAb	monoclonal antibody
MCP	major capsid protein
MOI	multiplicity of infection
mRNA	messenger RNA
NF-H1	neurofilament triplet H1-like protein
OIE	World Organisation for Animal Health (Office International des Epizooties)
ORF	open reading frame
PAb	polyclonal antibody
PCR	polymerase chain reaction
p.i.	post infection

PI	propidium iodide (stain)
PPIV	pike-perch iridovirus
qPCR	quantitative polymerase chain reaction
REA	restriction enzyme analysis
REV	rana esculenta virus
RNA	ribonucleic acid
rRNA	ribosomal RNA
RNase III	ribonuclease III
RNR-α	ribonucleotide reductase alpha subunit
RNR-β	ribonucleotide reductase beta subunit
RSIV	red sea bream iridovirus
SCRV	Santee-Cooper ranavirus
SERV	short-finned eel ranavirus
SGIV	Singapore grouper iridovirus
STIV	soft-shelled turtle iridovirus
TCID50	50% tissue culture infective dose
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
TGF-β	transforming growth factor beta
VHSV	viral haemorrhagic septicaemia virus
WSIV	white sturgeon iridovirus
40s	small ribosomal subunit 40s

1 Introduction

Ranaviruses belong to one of the five genera of the family *Iridoviridae* (Jancovich et al. 2011). They are large icosahedral viruses that infect a broad range of lower vertebrates (Williams et al. 2005). Ranaviruses have been associated with numerous disease outbreaks in natural and cultured populations of fish, amphibians and reptiles, and have become pathogens of both economic and ecological importance (Chinchar 2002, Chinchar et al. 2009). Several ranavirus isolates have been recovered from different host species in Australia, the Americas, Europe and Asia (Williams et al. 2005). In addition to the widely reported piscine die-offs, ranaviruses are also suspected to be partly responsible for the global declines of amphibian populations (Chinchar 2002). The World Organisation for Animal Health (OIE) recognizes one of the ranaviruses, epizootic haematopoietic necrosis virus (EHNV), as a notifiable pathogen for fish, whereas for amphibians all ranavirus infections are listed (OIE 2011). Ranaviruses cause cell death in a wide variety of fish, amphibian and mammalian cell lines, whereas in animals, the pathogenicity depends on the viral isolate as well as on the species, age and geographic origin of the host (Chinchar et al. 2005). Even though ranaviruses are somewhat host specific, some isolates have been shown to infect both fish and amphibians (Moody & Owens 1994, Mao et al. 1999a). In a host animal, ranaviruses cause systematic necrotizing infections, but subclinical and persistant infections may appear under certain conditions (Whittington & Reddacliff 1995, Whittington et al. 2010). Several studies have been published on the immune response to ranavirus infection in amphibians (Gantress et al. 2003, Maniero et al. 2006, Morales & Robert 2007, Morales et al. 2010), whereas the response elicited in piscine hosts is less well known.

The genome of ranaviruses is a single linear double-stranded DNA (dsDNA) molecule that is highly methylated, circularly permuted and contains terminal redundancies (Chinchar 2002, Jancovich et al. 2011). Currently, the complete genomic sequence of seven different ranaviruses has been determined (He et al. 2002, Jancovich et al. 2003, Song et al. 2004, Tan et al. 2004, Tsai et al. 2005, Huang et al. 2009, Jancovich et al. 2010). The most well-studied ranavirus gene is the major capsid protein (MCP), and the gene sequences of several ranavirus isolates are publicly available. MCP sequences have been used to study the evolution and phylogeny of ranaviruses, and PCR methods based on MCP gene have been developed for the detection of this group of viruses (Mao et al. 1996, Mao et al. 1997, Tidona et al. 1998, Hyatt et al. 2000, Marsh et al. 2002).

In this thesis, several ranaviruses, including previously characterised and some less well defined isolates, were studied on the basis of their gene sequences. The aim was to investigate the genomic differences between the isolates and to develop molecular methods for the detection and differentiation of ranaviruses. Additionally, the pathogenicity of ranaviruses to rainbow trout (*Oncorhynchus mykiss*) as well as the immune response to ranavirus infection in piscine epithelial cells was investigated.

2 Review of the literature

2.1 Genus *Ranavirus*

Ranaviruses belong to the genus *Ranavirus* of the family *Iridoviridae* with four other genera: *Iridovirus*, *Chloriridovirus*, *Lymphocystivirus* and *Megalocytivirus* (Jancovich et al. 2011). There are six official species in the genus *Ranavirus*: *Ambystoma tigrinum virus* (ATV), *Bohle iridovirus* (BIV), *Epizootic haematopoietic necrosis virus* (EHNV), *European catfish virus* (ECV), *Frog virus 3* (FV3), the type species of the genus *Ranavirus*, and *Santee-Cooper ranavirus* (SCRV) (Table 1). Within the species, separate isolates have been recognized by the International Committee on the Taxonomy of Viruses (ICTV). In addition, tentative species yet to be approved as members of the genus *Ranavirus* have been included to the recent ICTV report on ranavirus taxonomy (Jancovich et al. 2011).

Table 1. *Species and isolates in the genus* Ranavirus *(Jancovich et al. 2011).*

Species	Isolate
Ambystoma tigrinum virus	Ambystoma tigrinum virus (ATV)
	Regina ranavirus
Bohle iridovirus	Bohle iridovirus (BIV)
Epizootic haematopoietic necrosis virus	Epizootic haematopoietic necrosis virus (EHNV)
European catfish virus	European catfish virus (ECV)
	European sheatfish virus (ESV)
Frog virus 3	Frog virus 3 (FV3)
	Tiger frog virus (TFV)
Santee-Cooper ranavirus	Santee-Cooper ranavirus (SCRV)
	Largemouth bass virus (LMBV)
Tentative species	
Rana esculenta iridovirus (REIR)	
Singapore grouper iridovirus (SGIV)	
Grouper iridovirus (GIV)	
Testudo iridovirus (ThIV)	
Rana catesbeiana virus-Z (RCV-Z)	

2.1.1 Virion structure and cytopathology

Members of the family *Iridoviridae* are large double-stranded DNA (dsDNA) viruses. Virions are usually 120-200 nm in diameter and they display icosahedral symmetry (Chinchar 2002). The core of the virion consists of a nucleoprotein filament surrounded by a lipid membrane containing transmembrane proteins and a capsid composed of identical

capsomers (Jancovich et al. 2011) (Fig.1). The virions may acquire an envelope by budding through the host cell membrane. Even though enveloped virions possess a higher specific infectivity, both enveloped and non-enveloped virions are infectious (Braunwald et al. 1979).

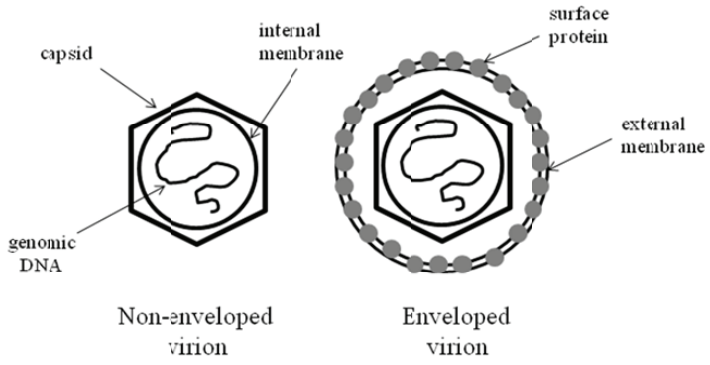


Figure 1. *Structure of non-enveloped and enveloped ranavirus virions.*

In addition to amphibians, fish and reptiles, ranaviruses are able to infect a wide variety of vertebrate cell lines including fish, amphibian and mammalian cells (Jancovich et al. 2011). Virus entry is mediated by currently unknown receptor, and occurs by one of two routes. Virions enter the host cell by either endocytosis (enveloped particles) or by uncoating at the plasma membrane (non-enveloped particles) (Chinchar 2002). Subsequent to uncoating, DNA cores are transported into the nucleus, where immediate early (IE) and delayed early (DE) viral transcripts are synthesised by cellular RNA polymerase II (Goorha et al. 1978, Goorha 1981). One of the transcripts, the viral DNA polymerase, catalyses the viral DNA synthesis in the nucleus (Goorha 1982, Chinchar 2002). The second stage of viral DNA replication occurs in the cytoplasm, where the newly synthesized viral DNA is transported and recombined into large, concatameric structures equivalent in size to more than ten genomes (Goorha 1982). The late viral mRNA transcription takes place in the cytoplasm or within viral assembly sites, and as with other DNA viruses, full late gene expression requires viral DNA synthesis (Chinchar 2002). No extensive post-translational processing nor glycosylation, sulfation or cleavage from precursors of ranavirus proteins has been detected (Chinchar et al. 2005).

High levels of methylation of cytosine at CpG residues in ranavirus DNA have been reported (Willis & Granoff 1980, Eaton et al. 1991). The viral DNA is methylated in the cytoplasm by virus-encoded cytosine DNA methyltransferase (Willis et al. 1984). Viral DNA methyltransferase has been molecularly characterized from FV3 and other iridoviruses (Kaur et al. 1995, Tidona et al. 1996, Mao et al. 1999b). It has been speculated that methylation is a protective mechanism preventing the nicking of viral

DNA by virus-encoded endonucleases (Goorha et al. 1984). On the other hand, at least one of the ranaviruses, Singapore grouper iridovirus (SGIV), lacks the DNA methyltransferase (Song et al. 2004), and this has evoked speculations that another role for the methylation of viral DNA may exist. Bacterial DNA containing unmethylated CpG residues has been shown to induce innate immunity following its interaction with toll-like receptor 9 (TLR9) (Bauer et al. 2001). It has been suggested that ranaviruses may methylate their genome in order to prevent the TLR9-mediated induction of innate immunity in the host (Williams et al. 2005).

Virion assembly occurs when viral structural proteins are transported into assembly sites and the concatameric viral DNA is packaged into virions via mechanisms that result in the generation of circularly permuted and terminally redundant genomes (Chinchar 2002). Virions exit the cell by budding trough the plasma membrane as enveloped particles or accumulate within the cytoplasm by forming large paracrystalline arrays (Chinchar 2002). The replication cycle of ranaviruses is summarized in Figure 2.

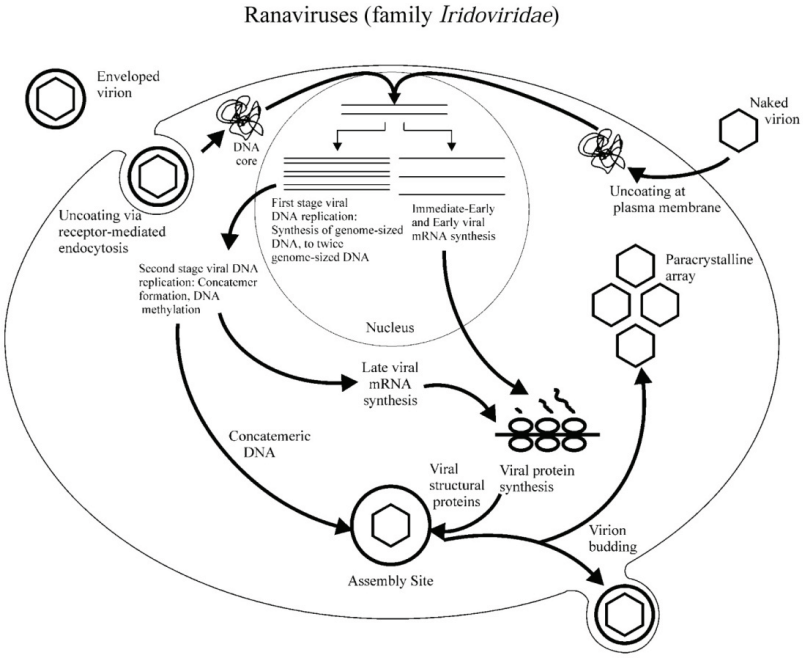


Figure 2. *Summary of the replication cycle of ranaviruses. Reprinted from Chinchar (2002) with the kind permission of Springer-Verlag Wien.*

FV3 replicates at temperatures between 12 and 32 ºC, and is inactivated within 30 min at temperatures higher than 55 ºC or by UV -irradiation (Chinchar 2002, Jancovich et al.

2011). Ranaviruses have been shown to maintain infectivity during one year of storage at +4 or -70 ºC (Bailey 2007). Some ranaviruses are sensitive to drying, whereas BIV reportedly survives up to 6 weeks of desiccation at 42 ºC (Jancovich et al. 2011). In cell culture, the replication of ranaviruses is rapid, and viral macromolecular synthesis and the cytopathic effect (CPE) can be detected within hours after infection (Goorha & Granoff 1974). As for the host cellular functions, ranavirus infection incurs swift inhibition of host cell DNA, RNA and protein synthesis and induces re-arrangement of the cellular structure (Murti et al. 1985, Willis et al. 1985, Chinchar 2002). Ranaviruses have also been shown to induce characteristic symptoms of apoptosis in host cells, such as chromatic condensation and DNA fragmentation (Zhang et al. 2001, Essbauer & Ahne 2002, Chinchar et al. 2003). Viral gene expression is not required in triggering apoptosis, but it is yet to be determined whether the induction of programmed cell death is mediated directly or indirectly by virion-associated proteins (Chinchar et al. 2003).

In the host, ranavirus infection involves haematopoietic tissues and other organs, such as the kidneys, liver, spleen and gastrointestinal tract, causing haemorrhages as well as focal or generalized necrosis (Reddacliff & Whittington 1996, Williams et al. 2005). Additional symptoms are haemorrhages and darkening of the skin, erratic swimming, anorexia, apathy and ataxia (Langdon 1989, Ogawa et al. 1990, Williams et al. 2005). In infected fish, viral particles can be detected in the cytoplasm of endothelial, epithelial and white blood cells of internal organs (Ahne et al. 1997).

2.1.2 Genomic properties of ranaviruses

The ranavirus genome is a single linear dsDNA molecule. So far, a complete genome sequence has been determined from seven ranavirus isolate: FV3 (Tan et al. 2004), EHNV (Jancovich et al. 2010), ATV (Jancovich et al. 2003), tiger frog virus (TFV, He et al. 2002), soft-shelled turtle iridovirus (STIV) (Huang et al. 2009), grouper iridovirus (GIV, Tsai et al. 2005) and Singapore grouper iridovirus (SGIV, Song et al. 2004). EHNV, SGIV and GIV were originally isolated from fish, FV3, ATV and TFV from amphibians and STIV is of reptilian origin.

The genome size of these isolates varied between 105 and 140 kbp, the GC content was 48-55% and the number of potential open reading frames (ORFs) was 92-139. The ORFs of the isolates ranged from 32 to over 1300 amino acids in length and they encode proteins involving in DNA replication and repair, transcription, nucleotide metabolism, virion structure and virus-host interactions. Examples of ranavirus proteins and their predicted functions are presented in Table 2. All of the genomes contained repetitive sequences, which have been suggested to have a role in gene regulation, transcription and protein function of viruses (Kashi & King 2006). Additionally, non-coding RNAs such as microRNAs (miRNAs) were identified in the STIV genome (Huang et al. 2009). In mammalian viruses, miRNAs participate in infection by inhibiting the host immune

response and apoptosis and by regulating gene expression and the viral life cycle (Nair & Zavolan 2006, Cullen 2007).

Table 2. *Examples of ranavirus-encoded proteins and their predicted functions as described in He et al.(2002), Tsai et al. (2005), Huang et al. (2009), and Jancovich et al. (2010).*

Function	Protein
DNA replication and repair	DNA polymerase
	Thymidine kinase
	NTPase/helicase
Transcription of DNA	RNA polymerase II
	Transcription elongation factor IIS
	Ribonuclease III (RNase III)
Nucleotide metabolism	Ribonucleotide reductase α and β-subunit (RNR-α and β)
	Thymidylate synthase (TS)
	Deoxyuridine trisphosphate nucleotidohydrolase (dUTPase)
Structural protein	Major capsid protein (MCP)
Virus-host interaction	3-Beta-hydroxy-delta 5-C27 steroid oxidoreductase-like protein
	Bcl-2-like protein
	LPS-induced tumor necrosis factor-alpha
	Eukaryotic initiation factor 2 (eIF-2α) -like protein

The main structural component of the ranavirus virion is the major capsid protein (MCP). The size of MCP is typically 50 kDa and it comprises 40-45% of the total virion protein (Williams 1996). The MCP gene is highly conserved within the family *Iridoviridae* and shares amino acid sequence identity with other viral families, such as *Asfarviridae*, *Ascoviridae* and *Phycodnaviridae* (Tidona et al. 1998, Chinchar et al. 2005). The MCP is one of the most studied genes of iridoviruses and it is considered to be a suitable target for the determination of viral evolution (Tidona et al. 1998). Based on the phylogenetic analysis of the complete genome sequences, EHNV, ATV, FV3, STIV and TFV are closely related to each other and cluster apart from the two grouper iridoviruses GIV and SGIV (Eaton et al. 2007, Huang et al. 2009, Jancovich et al. 2010).

2.1.3 Epidemiology and host specificity

Ranavirus-associated mortality and morbity has been reported among both farmed and wild fish populations in several countries around the world. Of the ranaviruses infecting fish, EHNV is the only species currently considered as notifiable by the World Organisation for Animal Health (OIE 2011). The European Union (EU) lists epizootic haematopoietic necrosis (EHN) as an exotic disease with potentially detrimental effects on aquaculture and wild aquatic animals (Anonymous 2006). EHNV was first isolated from redfin perch (*Perca fluviatilis*) and rainbow trout during disease outbreaks in Australia

(Langdon et al. 1986, Langdon et al. 1988). Subsequently, another closely related ranavirus isolate, ESV, was isolated from moribund sheatfish (*Silurus glanis*) fry of a commercial aquaculture unit with high mortalities in Germany (Ahne et al. 1989). In France and Italy, European catfish virus (ECV) was isolated from diseased catfish (black bullhead, *Ameiurus melas*, previously *Ictalurus melas*) (Pozet et al. 1992, Bovo et al. 1993). Another ranavirus, largemouth bass iridovirus (LBMV), was isolated following the mass mortality of wild largemouth bass (*Micropterus salmoides*) in South-Carolina, USA (Plumb et al. 1996). In South-East Asia, two ranavirus isolates, GIV and SGIV, were isolated from outbreaks among yellow grouper (*Epinephelus awoara*) (Lai et al. 2000) and brown-spotted grouper (*Epinephelus tauvina*) (Chua et al. 1994), respectively. Additionally, ranaviruses have been isolated from ornamental fish (Hedrick & McDowell 1995, Paperna et al. 2001, Gibson-Kueh et al. 2003). In addition to disease outbreaks, ranaviruses have been isolated from apparently healthy hosts such as pike-perch (*Sander lucioperca*, previously *Stizostedion lucioperca*) (Tapiovaara et al. 1998) and short-finned eel (*Anguilla australis*) (Bang Jensen et al. 2009).

Among amphibians, several ranavirus-inflicted disease outbreaks have occurred worldwide, and according to the OIE, all ranaviruses are notifiable pathogens for amphibians (OIE 2011). In addition to the pathogenic skin fungus *Batrachochytrium dendrobatidis*, ranaviruses are considered to be possible causative agents in the worldwide amphibian declines (Chinchar 2002). The first ranavirus isolated was FV3, originating from the kidney of a tumor-bearing northern leopard frog (*Lithobates pipiens*, formerly *Rana pipiens*) (Granoff et al. 1966). In 1992, BIV was isolated from the ornate burrowing frog (*Limnodynastes ornatus*) in Australia. Iridovirus-like agents were isolated from moribund green frogs (*Rana esculenta*) (Fijan et al. 1991) and from unusual mortalities of common frog (*Rana temporaria*) in the 1990s in Europe (Drury et al. 1995, Cunningham et al. 1996). ATV was isolated from disease outbreak among tiger salamanders (*Ambystoma tigrinum diaoboli*) in Canada (Bollinger et al. 1999). A FV3-like ranavirus was isolated from diseased pig frogs (*Rana grylio*) (Zhang et al. 2001), wood frogs (*Rana sylvatica*) and northern leopard frogs (Greer et al. 2005). In South America, a ranavirus closely related to FV3 and ATV caused mortality and morbidity in frogs (*Atelognathus patagonicus*) (Fox et al. 2006). In Venezuela, seven different ranavirus isolates were recovered from toads (*Bufo marinus*) and frogs (*Leptodactylus* sp.) (Zupanovic et al. 1998). Recently, reports have been published of ranavirus-inflected outbreaks in red-tailed knobby newts (*Tylototriton kweichowensis*) in Belgium and the Netherlands (Pasmans et al. 2008), in common midwife toads (*Alytes obstetricians*) and alpine newts (*Mesotriton alpestris*) in Spain (Balseiro et al. 2009a, Balseiro et al. 2009b), and in edible frogs (*Pelophylax* kl. *esculentus*) in Denmark (Ariel et al. 2009a).

In reptiles, ranaviruses have been isolated from several species, such as diseased green pythons (*Chondropython viridis*) (Hyatt et al. 2002), leaf-tailed geckos (*Uroplatus fimbriatus*) (Marschang et al. 2005), and various turtles and tortoises (Chen et al. 1999, Marschang et al. 1999, De Voe et al. 2004, Allender et al. 2006, Johnson et al. 2007, Johnson et al. 2008).

Ranaviruses are able to infect fish, amphibians and reptilians, but the isolates show marked specificity regarding the species, age and geographical origin of the host animal (Chinchar 2002, Chinchar et al. 2005). In addition, the route of transmission, viral dosage and environmental factors play a role in the onset of the disease (Brunner et al. 2005, Brunner et al. 2007). So far, EHNV has only been isolated in Australia, and it causes mortality in different ages of Australian stocks of rainbow trout, redfin perch and other native teleost species (Langdon et al. 1988, Langdon 1989, Whittington et al. 1994, Whittington & Reddacliff 1995). However, in European specimens of redfin perch and rainbow trout, significant mortality was only observed in fish challenged by intraperitoneal (IP) injection, and not in fish infected by bath or cohabitation (Ariel & Bang Jensen 2009). It has been shown with common frogs (*Rana temporaria*) that ranavirus can be transmitted from infected to naïve animals via a common water source (Bailey & Feist 2011). Furthermore, EHNV has been shown to be pathogenic to pike (*Esox lucius*), pike-perch and black bullhead in certain experimental conditions (Bang Jensen et al. 2009, Gobbo et al. 2010, Bang Jensen et al. 2011a). Both ECV and ESV cause mortality in juvenile and adult fish (Ahne et al. 1990, Pozet et al. 1992, Bovo et al. 1993, Gobbo et al. 2010), even though with ESV, mortality in adults is markedly lower than in juveniles (Ahne et al. 1991). Despite the fact that ECV and ESV are very closely related, they seem to be host specific. In a bath challenge with black bullhead, mortality was observed with ECV but not with ESV (Gobbo et al. 2010). Additionally, ECV and ESV caused mortality in IP-challenged pike-perch (Bang Jensen et al. 2011a), whereas pike have been found to be susceptible to ESV but not to ECV in bath challenge (Bang Jensen et al. 2009). Pike-perch iridovirus (PPIV) and short-finned eel ranavirus (SERV) caused mortality in pike, but not in pike-perch (Bang Jensen et al. 2009, Bang Jensen et al. 2011a). LBMV caused mortality in wild adult largemouth bass (Plumb et al. 1996), whereas in experimental conditions mortality was observed in IP- but not orally challenged juvenile fish (Zilberg et al. 2000, Woodland et al. 2002).

Adult *Xenopus laevis* individuals are able to resist FV3 infection, whereas the isolate is highly pathogenic to tadpoles (Gantress et al. 2003, Robert et al. 2005). In Canada, FV3-like ranavirus isolate has been reported to infect tadpoles, larval salamanders and adult newts of sympatric amphibian species (Duffus et al. 2008). BIV is highly pathogenic to tadpoles and juveniles of several amphibian species (Cullen & Owens 2002), and it has been shown to also cause mortality in fish (Moody & Owens 1994, Ariel & Owens 1997). Another example of a ranavirus infecting both fish and amphibians was reported in Redwood Creek, USA, where the same FV3-like isolate was found from moribund threespine sticklebacks (*Gasterosteus aculeatus*) and diseased red-legged frog (*Rana aurora*) tadpoles (Mao et al. 1999a). In challenge studies, amphibian ranavirus FV3 did not cause mortality in different piscine hosts (Bang Jensen et al. 2009, Gobbo et al. 2010, Bang Jensen et al. 2011a). Additionally, common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) were challenged with 8 different piscine and amphibian ranaviruses with non-significant mortalities (Bang Jensen et al. 2011b). On the other hand, Picco et al.

(2010) were able to isolate virus from largemouth bass IP injected with tiger salamander ranavirus, even though the fish displayed no signs of disease.

Temperature seems to affect the survival of the host species during ranavirus infection. Whittington and Reddacliff (1995) demonstrated that the incubation period in EHVN infection in redfin perch decreased with increasing water temperature, and no infection was detected below 12ºC. A similar trend was not seen in rainbow trout, even though the incubation time did increase at lower temperatures. Salamanders exposed to ATV survived at 26 ºC, whereas at lower temperatures (18 and 10 ºC), high mortalities were observed (Rojas et al. 2005).

2.2 Detection and identification of ranaviruses

2.2.1 Virus isolation in cell culture

Virus isolation in cell culture is the gold-standard method for the detection of viruses in fish. According to the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE 2009, 2012), for detecting EHNV in fish or ranaviruses in amphibians, the tissues of the host animal (e.g. kidney, liver and spleen) are homogenized into appropriate medium and centrifuged. The clarified tissue homogenate (supernatant) is then inoculated into almost confluent cell culture monolayers of bluegill fry (BF-2), fathead minnow (FHM), epithelioma papulosum cyprini (EPC) or Chinook salmon embryo (CHSE-214) cells and incubated for 6 days at 15-22 ºC, depending on the cell line used. The primary culture is then frozen and thawed, and the culture supernatant is inoculated into fresh cells to obtain secondary culture. Both primary and secondary cultures are observed twice during the incubation period for cytopathic effect (CPE) caused by virus infection. CPE induced by EHNV consists of focal lysis surrounded by granular cells, and the change in the cells can extend to the entire monolayer, causing detachment and disintegration (OIE 2009).

EU legislation includes a standard protocol for virological examination of fish viruses in cell culture (Anonymous 2001). In the protocol, two cell lines are used: either BF-2 or rainbow trout gonad (RTG-2) cells and EPC or FHM cells. The cells are inoculated with tissue homogenate supernatant and incubated for 7 to 10 days at 15 ºC in two consecutive cultures.

2.2.2 Electron microscopy

Due to their small size, viruses can often only be visualized by electron microscopy (EM). EM has been an important method in the discovery of new viruses, diagnosis of viral infections and investigations of virus-host interactions (Roingeard 2008). Likewise in

identification of ranaviruses and other iridoviruses, the detection of the icosahedral-shaped virions by EM has been and still is a commonly used practice (Ahne et al. 1989, Eaton et al. 1991, Tapiovaara et al. 1998, Zupanovic et al. 1998, Qin et al. 2001, Drury et al. 2002, Qin et al. 2003).

2.2.3 Immunochemical and protein-based methods

Mammals or fish do not produce neutralizing antibodies against ranaviruses (OIE 2009). Hence, neutralization tests cannot be used in the detection of EHNV or other ranaviruses and the immunochemical identification methods are based on the detection of viral epitopes by anti-ranavirus antibodies. Hyatt et al. (1991) described protocols for the detection of EHNV by antigen-capture ELISA, immunohistochemistry (IHC) and immune-electron microscopy. An improved ELISA method was published two years later by Whittington and Steiner (1993). Hedrick et al. (1992) described an indirect fluorescent antibody test (IFAT) for the detection of ranaviruses. Western blotting has been used in the identification of EHVN and BIV (Hengstberger et al. 1993). Several studies have shown that different ranavirus isolates cross-react in polyclonal antibody-based methods (Ahne et al. 1998, Hyatt et al. 2000, Bang Jensen et al. 2009, Gobbo et al. 2010), and these methods can therefore only be used in detection, not in the differentiation of ranaviruses.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-page, has been used to compare ranavirus polypeptides (Mao et al. 1997, Ahne et al. 1998), but no specific signature poplypeptides distinguishing isolates from each other have been detected (Hyatt et al. 2000).

2.2.4 Restriction enzyme analysis, PCR and sequencing

The detection and differentiation of ranaviruses based on viral genomic DNA has been described in many publications. Hengstberger et al. (1993) studied the differences between EHNV and BIV by restriction enzyme profiles of their genomic DNA. Differences in the restriction patterns were detected between isolates using *Kpn*I, *Bam*HI, *Hind*III and *Nco*I enzymes. The same approach has been applied later with different enzymes and several other ranavirus isolates (Mao et al. 1997, Ahne et al. 1998, Bollinger et al. 1999, Hyatt et al. 2000). In addition to genomic restriction analysis, Southern blotting and hybridization with a labelled ranavirus DNA- probe have been used in finding differences between isolates (Ahne et al. 1998, Hyatt et al. 2000).

Polymerase chain reaction (PCR) was first utilized to detect EHNV and BIV by amplifying a fragment of an unknown region of the ranavirus genome with primers described by Gould et al. (1995). In 1996, the first ranavirus nucleotide sequence was published when cloning and sequencing of the complete MCP gene of FV3 was accomplished (Mao et al. 1996). The determination of gene sequences from PCR-

amplified fragments of other ranaviruses soon followed (Mao et al. 1997, Mao et al. 1999a, Mao et al. 1999b), and the accumulating amount of sequence data lead to the development of new methods for ranavirus detection. Hyatt et al. (2000) published PCR primers amplifying the complete ranavirus MCP gene in three overlapping fragments, and described the distinguishing of several ranaviruses based on the nucleotide differences in the MCP gene sequence. Marsh and colleagues (2002) published a method based on a combination of two different PCR assays and subsequent restriction enzyme analysis (REA) of a partial MCP gene for the differentiation of EHNV, BIV, FV3, Wamena virus (WV), Gutapo virus (GV) and ECV/ESV. In addition to the MCP gene, other ranavirus genes, such as DNA methyltransferase (Kaur et al. 1995, Mao et al. 1999b), eIF-2α (Essbauer et al. 2001), ATPase (Sudthongkong et al. 2002, Wang et al. 2003), DNA polymerase (Hanson et al. 2006), and thymidine kinase (Coupar et al. 2005, Zhao et al. 2009), have also been studied. Amplified fragment length polymorphism (AFLP), a technique that uses both restriction enzymes and PCR, has been used to determine the variation between different isolates of largemouth bass virus (LMBV) (Goldberg et al. 2003). For the detection and quantitation of LBMV, quantitative real-time PCR (qPCR) methods based on the MCP gene have been developed (Goldberg et al. 2003, Getchell et al. 2007). Pallister et al. (2007) developed a real-time PCR method based on MCP and a variable genomic region for the detection and differentiation of EHNV, BIV and ECV/ESV.

MCP remains the most widely used gene both in detection and phylogenetic studies of ranaviruses, especially in the description of newly found isolates (Pasmans et al. 2008, Ariel et al. 2009a, Balseiro et al. 2009a, Balseiro et al. 2009b). Complete genome analysis discussed in section 2.1.2 is becoming more prevalent, but is not at the moment regularly used for diagnostic purposes.

2.2.5 Diagnostic methods recommended by the OIE

The current OIE Manual of Diagnostic Tests for Aquatic Animals includes several methods for detecting epizootic haematopoietic necrosis (EHN, OIE 2009) in fish and infection with ranavirus in amphibians (OIE 2012). Field diagnostic and clinical methods comprise observation of clinical signs and behavioural changes as well as cross and microscopic pathology. Direct methods for pathogen detection, such as light, electron and negative contrast electron microscopy, are mentioned. The manual contains the following methods for virus isolation and identification: cell culture isolation, immunoperoxidase staining, antigen-capture ELISA, immunoelectron microscopy, immunohistochemistry or molecular techniques. The molecular techniques include PCR and REA by Marsh et al. (2002) and an alternative PCR method by Hyatt et al. (2000) followed by sequencing analysis. Both of these molecular methods have been described in section 2.2.4.

2.3 Host immune response

All organisms, including fish and other lower vertebrates acting as hosts to ranaviruses, are constantly exposed to pathogens in their environment. The need to differentiate between "self" and "non-self" has led to the evolution of a defensive immune system consisting of a variety of molecules, cells and tissues. The immune system of lower vertebrates is fundamentally similar to that of mammals and can be divided into innate and adaptive systems, both types including cellular and humoral responses (Magor & Magor 2001, Bone & Moore 2008). However, evidence from both fish and mammalian immunology shows that this dichotomy is artificial and that innate and adaptive immunity are combinational rather than two separate systems (Flajnik & Du Pasquier 2004, Magnadóttir 2006). Cellular responses involve cells that engulf and destroy or otherwise attack pathogens, whereas humoral responses include responses involving antibodies or any defensive molecules that are delivered, for instance, via blood, interstitial fluids or exocrine secretions (Bone & Moore 2008).

2.3.1 Innate immunity

Innate immunity is the first line of immune defence and it activates and regulates the adaptive response and is involved in the maintenance of homeostasis (Fearon 1997). Innate immunity is non-specific, acting against many different types of pathogens and repeated exposure to certain types of pathogens does not increase the efficacy of the response (Bone & Moore 2008). In addition to humoral and cellular responses, innate immunity includes physical barriers such as the skin or mucosal surfaces (Ellis 2001, Freihorst & Ogra 2001). The humoral components of innate immunity include growth inhibitors, various lytic enzymes and components of the complement pathways, agglutinins and precipitins, natural antibodies, cytokines, chemokines and antibacterial peptides (Magnadóttir 2006). Innate immune recognition is mediated by germline encoded pattern-recognition receptors (PRRs) that have broad specificities for conserved and invariant features of microorganisms, so called microbe-associated molecular patterns (MAMPs) (Medzhitov 2007, Fritz et al. 2008).

Of the several functionally distinct classes of PRR, toll-like receptors (TLRs) are the best characterized (Medzhitov 2007). TLRs are type I integral membrane glycoproteins and they recognize distinct microbial components such as lipopolysaccharides, lipopeptides and other proteins of bacteria, fungus and parasites, as well as viral nucleic acids (Akira et al. 2006). TLR recognition elicits intracellular signalling cascades that rapidly induce inflammatory and antimicrobial responses (Medzhitov 2007). Among members of the TLR family, TLR3, TLR7, TLR8 and TLR9 are involved in the recognition of viral nucleotides, which usually induces type I interferon (IFN) production activating target cells in both an autocrine and paracrine manner (Akira et al. 2006). Additionally, TLR-mediated intracellular signalling leads to up-regulation of transcription factor NF-κβ, which in turn regulates the expression of cytokines, growth factors and effector enzymes

(Hayden & Ghosh 2004). Pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) coordinate local and systemic inflammatory responses, and they elicit processes allowing serum proteins and leukocytes to be recruited to the site of infection (Medzhitov 2007). In this cascade, TNF-α is normally the first cytokine to be secreted, which leads to the downstream expression of IL-1β and chemokines such as IL-8 (Secombes et al. 2009). In contrast to mammals, TNF-α is the only member of the TNF superfamily that has been found in fish, even though two TNF-α homologues have been isolated in rainbow trout and carp (Zou et al. 2002, Saeij et al. 2003, Goetz et al. 2004). The similarity of TNF-α gene sequences in mammals is reasonably high, whereas in fish the identity is generally lower (Goetz et al. 2004). TNF-α has an important role in different cellular responses, including leukocyte activation and migration, cell proliferation, differentiation and apoptosis (García-Ayala & Chavez-Pozo 2009). There are two distinct cell-surface receptors expressed in virtually all cell types that are known to interact with TNF-α (Goetz et al. 2004). Binding of TNF-α to one of the receptors, TNFR1, initiates a signal pathway leading to apoptosis (dos Santos et al. 2008). Apoptosis is also mediated by another antiviral cytokine, interferon γ (IFN-γ), which is in turn regulated by other cytokines such as TNF-α, IL-1β and IL-4 (Boehm et al. 1997). IL-1β is a multifunctional cytokine affecting several cell types by acting as a signalling molecule between the immune system and other internal systems, and it often functions in concert with other cytokines or small mediator molecules (Dinarello 1996, Engelsma et al. 2001). IL-1β belongs to the superfamily of pro-inflammatory cytokines, ligands and receptors associated with acute and chronic inflammation, and it is the only IL-1 superfamily cytokine homologue identified in fish genome so far (Bird et al. 2002, Dinarello 2011). Teleost IL-1β sequences share roughly 25-30% identity with mammalian IL-1β, and even among fish species the percentage identity in IL-1β sequences is remarkably low (Engelsma et al. 2002). Because the immune and inflammatory responses can have damaging effects in the host, limiting the immune response is just as crucial for the host as protection against the infecting pathogen (Medzhitov 2007). Some of the cytokines, including IL-10 and transforming growth factor β (TGF-β), have regulatory effects. IL-10 was initially described as a cytokine synthesis inhibitory factor, but is currently known to be a multifunctional cytokine that regulates immunity and inflammatory responses (Savan et al. 2003). IL-10 inhibits the activation of macrophages/monocytes, thus inhibiting nitric oxide (NO) production and the expression of costimulatory molecules and the synthesis of several cytokines, including TNF-α and IL-1β (Moore et al. 2001, Savan et al. 2003). The presence of IL-10 has been confirmed in the genomes of several teleosts, including two puffer fish species (Lutfalla et al. 2003, Zou et al. 2003), carp (Savan et al. 2003) and rainbow trout (Inoue et al. 2005). TGF-β is a pleiotropic cytokine that controls the initiation and resolution of inflammatory responses by regulating tissue remodelling, proliferation, differentiation, chemotaxiss, activation and survival of several leukocyte types (Li et al. 2006). Homologues of TGF-β1, the TGF-β isoform being predominantly expressed in the immune system, have been identified in several teleosts (Hardie et al. 1998, Harms et al. 2000, Yin & Kwan 2000, Tafalla et al. 2003, Haddad et al. 2008).

2.3.2 Adaptive immunity

Adaptive immunity produces a more efficient response to specific antigen molecules, and the recognition is mediated by T and B cell receptors and antibodies produced by B cells (Medzhitov 2007, Murphy et al. 2008). Adaptive immunity is also capable of generating immunological memory towards specific pathogens (Murphy et al. 2008). Both antigen receptors and antibodies are encoded by genes that are assembled from variable and constant fragments through somatic recombination coordinated by RAG (recombination-activating gene) –proteins (Sadofsky 2004). T cells can be defined by the receptors they carry on the cell surface: T-helper cells express co-receptor CD4, whereas cytotoxic T lymphocytes (CTLs) carry the CD8 receptor (Medzhitov 2007). With the CD8 receptor, CTLs recognize antigenic peptides bound to major histocompatibility complex (MHC) class I molecules, whereas T-helper cells interact with MHC class II molecules (Murphy et al. 2008). The MHC class I antigen presentation pathway plays an important role in the immune response against viral infections (Hewitt 2003). MHC class I molecules are heterodimers of a heavy (α) chain, a transmembrane glycoprotein and a β2-microglobulin (β2M) protein (Murphy et al. 2008). β2M has an important role in the correct folding and cell surface expression of the MHC class I molecules (Vitiello et al. 1990, Solheim 1999). MHC class I molecules are expressed on the cell surfaces of all nucleated cells and they collect peptides derived from proteins synthesized in the cytosol, including viral proteins, and present them at the cell surface (Hewitt 2003). Virus- specific CTLs monitor MHC class I molecules bound to viral peptides and eliminate infected cells via perforin- or granzyme- mediated killing (Vitiello et al. 1990, Hewitt 2003, Murphy et al. 2008). Several reports of cellular equivalents of $CD8^+$ cytotoxic cells and MHC molecules in fish have been published (Hansen et al. 1999, Litman et al. 1999, Hansen & Strassburger 2000, Fischer et al. 2003, Secombes et al. 2005, Fischer et al. 2006, Yang et al. 2006, Chen et al. 2010). Likewise, cytotoxicity mediated by peripheral blood lymphocytes has been shown to be involved in the immune responses of virus-infected fish cells (Zhou et al. 2001, Somamoto et al. 2002). In amphibians, CTL activity of the African clawed frog (*Xenopus laevis*) in antigen-specific immune responses *in vitro* have been reported (Robert et al. 2002).

2.3.3 Ranaviruses and host immune response

Immune responses to FV3 infection in *Xenopus laevis* have been described in several studies. Adaptive antiviral responses were observed when after secondary infection with FV3, adult *Xenopus* cleared virus more rapidly and generated anti-FV3 IgY antibodies (Gantress et al. 2003). Maniero et al. (2006) demonstrated that *Xenopus* developed long-lasting protective humoral immunity after initial exposure to FV3. MHC class I-restricted $CD8^+$ CTLs were shown to be critically involved in the recognition and clearance of FV3 infection (Morales & Robert 2007). According to Morales et al. (2010), FV3 infection induced an immediate increase in peritoneal leukocytes and mononucleated macrophage-like cells, later followed by an increase in natural killer (NK) cells and T cells.

Additionally, rapid up-regulation of pro-inflammatory genes, such as arginase 1, IL-1β and TNF-α, was detected. Antimicrobial peptides (AMPs) are molecules with inhibitory activity against bacteria, fungi and viruses, and have been isolated from various organisms, including mammals, fish, amphibians and insects (Silphaduang & Noga 2001, Zasloff 2002). AMPs have been shown to have a potential role in protecting amphibians and fish from FV3 infection (Chinchar et al. 2001, Chinchar et al. 2004).

The immune response of piscine hosts to ranavirus infection has been less studied. SGIV induced the transcription of immune genes, including interferon regulatory factor 1, interleukin 8 and TNF-α in grouper (*Epinephelus* spp.) spleen cells, and the transcription was shown to be regulated by stress-activated protein kinases (SAPKs) (Huang et al. 2011a). Increased phosphorylation of SAPKs and activation in SAPK signalling pathways were detected after infection with SGIV. One of the aims of this study was to further elucidate the immune processes activated in fish cells during ranavirus infection.

3 Aims of the study

The general aim of this study was to develop methods for the detection and differentiation of ranaviruses and to study the host immune responses against ranavirus infection. The specific aims were:

1. To develop new PCR and restriction enzyme analysis (REA) methods for the detection and differentiation of ranaviruses and to obtain novel sequence data from known and previously uncharacterized ranavirus isolates.

2. To compare ranavirus-like isolates originating from cod (*Gadus morhua*) and turbot (*Psetta maxima*) to other ranaviruses with respect to their reactivity in IFAT, genetic properties and pathogenicity.

3. To develop a quantitative real-time PCR method (qPCR) for the detection and quantitation of ranaviruses and to apply the method to infected cell culture and fish tissue samples.

4. To study the initial immune responses against ranavirus infection in a fish epithelial cell culture model.

4 Materials and methods

4.1 Materials

4.1.1 Viral isolates

Several ranavirus isolates were used in this study (Table 3). In addition, two non-ranaviruses were used in publication **III**: koi herpesvirus (KHV) and DNA from red sea bream iridovirus (RSIV).

Table 3. *Ranavirus isolates studied in this thesis.*

Virus	Acronym	Host	Reference	Publication
Bohle iridovirus	BIV	Burrowing frog *Limnodynastes ornatus*	(Speare & Smith 1992)	**I**, **II**
Cod iridovirus	CodV	Cod *Gadus morhua*	(Jensen et al. 1979)	**II**
Doctor fish virus	DFV	Doctor fish *Labroides dimidatus*	(Hedrick & McDowell 1995)	**I**, **II**, **III**, **IV**
Epizootic haematopoietic necrosis virus	EHNV	Redfin perch *Perca fluviatilis*	(Langdon et al. 1986)	**I**, **II**, **III**, **IV**
European catfish virus	ECV	Black bullhead *Ameiurus melas*	(Pozet et al. 1992, Bovo et al. 1993)	**I**, **II**, **III**, **IV**
European sheatfish virus	ESV	Wels catfish *Silurus glanis*	(Ahne et al. 1989)	**I**, **II**, **III**
Frog virus 3	FV3	Northern leopard frog *Lithobates pipiens*	(Granoff et al. 1966)	**I**, **II**, **III**, **IV**
Guppy virus 6	GV6	Guppy *Poecilia reticulata*	(Hedrick & McDowell 1995)	**I**, **III**
Pike-perch iridovirus	PPIV	Pike-perch *Sander lucioperca*	(Tapiovaara et al. 1998)	**I**, **II**, **III**
Rana esculenta virus Italy 282/02	REV 282/I02	Edible frog *Pelophylax esculentus*	**I**	**I**
Ranavirus maxima DK 9995205	Rmax	Turbot *Psetta maxima*	**II**	**II**
Short-finned eel ranavirus	SERV	Short-finned eel *Anguilla australis*	(Bang Jensen et al. 2009)	**I**, **II**, **III**
Singapore grouper iridovirus	SGIV	Grouper (*Epinephelus* sp.)	(Chua et al. 1994, Chou et al. 1998)	**I**

4.1.2 Cell culture lines

Two cell lines were used in virus propagation and isolation; epithelioma papulosum cyprini (EPC) (**I**, **III**, **IV**) and bluegill fry (BF-2) (**II**, **III**) cells.

The EPC cell line was initially established from proliferative skin lesions of common carp (*Cyprinus carpio*) (Fijan et al. 1983). However, it was recently reported that the current lineages of EPC cells have been contaminated and ultimately replaced by fathead minnow (*Pimephales promelas*) epithelial-like cells (Winton et al. 2010). Accordingly, the EPC cells used here were confirmed to be fathead minnow cells in study **IV**. Despite the newly revealed species of origin of the cell line, the "EPC" designation has remained in use. The American Type Culture Collection (ATCC) currently provides these cells designated as EPC (epithelioma papulosum cyprini, ATCC accession number CRL-2872), and with the following description: fathead minnow (*Pimephales promelas*), source organ: skin, cell type: epithelial. Additionally, another epithelial cell line of fathead minnow, FHM (Gravell & Malsberger 1965), exists, also provided by the ATCC (accession number CCL-42). However, the cellular morphology of EPC and FHM cells is not identical (Winton et al. 2010), and these two cell lines reportedly vary in their susceptibility to a range of viruses (Lorenzen et al. 1999, Ariel et al. 2009b). Therefore, distinction of these two fathead minnow cell lines is necessary.

BF-2 cells are fibroblasts that originate from the caudal trunk of bluegill (*Lepomis macrochirus*) (Wolf et al. 1966). In addition to BF-2 cells, striped snake head cells (SSN-1) (Frerichs et al. 1991) were used in the isolation of Rmax from turbot fry in study **II**.

4.1.3 Fish used in the challenge trials

In study **II**, rainbow trout fry were used to test the pathogenicity of a panel of ranavirus isolates. Rainbow trout is a fish species of great economic importance, and the OIE categorises it with redfin perch as one of the two teleost species susceptible to EHNV-infection in natural conditions (OIE 2009). The average weight of the fry was 1.5 g, and they were obtained from a certified virus-free hatchery in Rønhøjgård, Denmark. The fry were screened for viral, parasitic and bacterial infections prior to challenge.

In study **III**, selected samples from a challenge study on pike fry published earlier by Bang Jensen et al. (2009) were used. The brood stock consisted of wild fish from the Danish lake Langesø in the central part of Sealand. At the time of challenge, the average weight of the fry was 0.03 g.

4.2 Methods

4.2.1 Propagation of ranaviruses in cell cultures

In publications **I**, **III** and **IV**, the ranavirus isolates were grown in EPC cells at room temperature (22ºC) according to the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE 2006, 2009, 2012). The cells were collected when the cytopathic effect (CPE) was complete.

In publication **II**, Rmax was isolated from turbot fry with no clinical signs using cell cultures. The tissue homogenate of turbot fry was diluted 1:10 in cell culture medium. The suspension was centrifuged at 4000 x g and the resulting supernatant was inoculated onto BF-2 and SSN-1 cells at two dilutions: 10^{-1} and 10^{-2}. In addition to Rmax, ten other isolates (CodV, EHNV, ECV(It), ECV (Fr), ESV, FV3, PPIV, REV 282/I02 and SERV) were propagated in BF-2 cells. The cells were grown at 20 ºC in Eagle's MEM supplemented with 10% foetal bovine serum, 1% glutamine, 100 IU/ml penicillin and 40 g/ml streptomycin. After inoculation with virus, the cells were incubated at 15ºC until the CPE was complete. Afterwards, the cells were frozen and thawed three times and the cell debris was separated from the viral suspension by centrifugation at 4000 x g. The viral suspensions were stored at – 80 ºC.

In order to determine the titre of the propagated ranavirus isolates, BF-2 (**II**) or EPC (**III**, **IV**) cells were grown in 96-well plates (CellBind, Corning, MA, USA). The cells were inoculated with the isolate in question with ten-fold dilutions, six (II) or eight (**III**, **IV**) replicates per dilution, and the 50% tissue culture infective dose ($TCID_{50}$) was calculated according to Reed and Muench (1938).

In publication **III** and **IV**, samples from the same cell culture experiment were studied. Approximately 800 000 EPC or BF-2 cells were seeded in 12-well plates (CellBind) and infected with EHNV, ECV, FV3 or DFV with a multiplicity of infection (MOI) of 2.5. Cells from duplicate wells were collected 1, 6, 24, 36, 48 and 72 h after infection. Duplicate negative control samples were collected either at the end of the study period (**III**) or at each time point studied (**IV**). The cells were collected after removal of the cell culture medium by scraping them into either PBS (**III**) or RLT -buffer (RNaeasy Mini Kit, Qiagen, Valencia, CA, USA, **IV**). In publication **III**, viral quantities were determined from both EPC and BF-2 cells. In publication **IV**, mRNA expression of selected immune genes was analysed from EPC cells.

4.2.2 Immunostimulation of EPC cells

In publication **IV**, EPC cells with a high level of gene expression were generated by growing approximately 800 000 cells/well in 12-well plates and incubating them with

either lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8 (30 µg ml-1; Sigma-Aldrich, St. Louis, MO, USA), CpG-ODN 1585 (GGTCAACGTTGA; 2 µM; InvivoGen, San Diego, CA, USA) or poly I:C (10 µg ml-1; Sigma-Aldrich). Both LPS and CpG-ODNs (synthetic oligonucleotides containing unmethylated CpG motifs) are used as bacterial mimics, whereas poly I:C, a synthetic analogue for double-stranded RNA, is used to mimic the viral genome. Replicate wells were sampled at 6, 24 and 48 h for each stimulant. The gene expression levels were studied from duplicate wells for each time point. The remaining stimulated cells were pooled and used to generate a standard cDNA. The standard cDNA was used to assess qPCR assay efficiency and as a calibrator sample in each assay plate.

4.2.3 Challenge trials

The challenge trials (**II** and **III**) were carried out in the challenge facilities of the National Veterinary Institute, Århus, Denmark. The challenge trials were designed and performed according to the Danish legislation on the use of animals in experimental trials, under a licence (no: 2007/561-1312) given by Dyreforsøgstilsynet (the Danish Ministry of Justice for Animal Experiments Inspectorate).

In publication **II**, the rainbow trout fry were kept in 10-litre tanks with 50 animals per tank in 15 ºC water with constant aeration and a flow-through system. The fry were tested in three challenge treatments: intraperitoneal inoculation of 50 µl viral suspension (10^4 $TCID_{50}$) per fish, bath exposure at a high dose (10^4 $TCID_{50}$/ml) and at a lower dose (10^3 $TCID_{50}$/ml). Nine ranavirus isolates (BIV, CodV, EHNV, ECV(Fr), ECV(It), ESV, FV3, PPIV and Rmax) were tested in the three treatments. A negative and positive control was included in each treatment. The negative control consisted of cell culture medium, while the positive control contained viral haemorrhagic septicaemia (VHS) virus strain DK-3592B (Lorenzen et al. 1993). Fish were tranquilised with benzocaine prior to IP inoculation. In bath exposure, the water levels were lowered and circulation was stopped for 2 h. Aeration of water was maintained during this time. Fish were monitored and fed twice daily. Mortality was recorded daily, and moribund or dead fish were collected and frozen at -20 ºC for virological examination at the end of each trial. Five fish were collected from each tank 7 and 28 days post-challenge and examined individually.

In publication **III**, viral quantities in fish tissues were studied. Selected samples from an EHNV challenge experiment on pike fry carried out by Bang Jensen et al. (2009) were analysed with quantitative PCR (qPCR). The conditions of the experiment were the following: pike fry of an average weight of 0.03 g were bath challenged in aquaria in two different water temperatures, 12 °C and 20 °C. The fish were exposed for 2 h to EHNV at a titre of 10^4 $TCID_{50}$/ml in duplicate aquaria. A negative control group was included, with fish bath-challenged with cell culture media. Dead fish were removed daily from each aquarium, pooled and frozen at -80 °C. After 28 days the surviving fish were euthanised and sampled for virological examination. Altogether, 14 samples of pooled fish from

different time points of infection were selected for examination with qPCR (Table 4). In addition, 2 samples from negative control fish were tested. Before DNA extraction, the fish were homogenized according to Commission Decision 2001/183/EC (Anonymous 2001).

Table 4. *The EHNV-challenged pike fry analysed with qPCR in publication **III**. The number of fish pooled in each sample is given for each time point.*

Treatment	Time of sampling, days after infection									
	1	3	5	7	9	11	13	15	16	17
Tank 1 12 ºC	-	1	2	3	6	2	5	-	1	-
Tank 2 12 ºC	-	-	-	2	5	4	12	-	-	-
Tank 3 22 ºC	17	4	-	-	-	-	-	-	1	-
Negative control	-	-	-	-	-	-	-	1	-	1

4.2.4 Virus re-isolation

Fish sampled in the challenge trial in publication **II** were homogenized using a mortar, pestle and sterile sand. The samples were diluted with 2 ml sterile EMEM with 10% newborn calf serum, TRIS-buffer and penicillin (100 U/ml)/streptomycin (100 µg/ml) and centrifuged at 4000 x g at 5 ºC for 20 min. For each sample, 25 µl/ml Hexamycin was added to the supernatant and the samples incubated at 5 ºC overnight or at 15 ºC for 4 h. The samples were then inoculated into 24-h-old EPC cells in 24-well trays, at final dilutions of 1:100, 1:1000 and 1: 10 000. The inoculated cell cultures were incubated at 20 ºC for 1 wk, with regular examination for cytopathic effect (CPE). After one week, samples with no CPE were re-inoculated into a new culture of EPC cells. After two weeks, the sample was considered negative if no CPE was detected. Cell cultures with a positive CPE were stored at -80 ºC.

4.2.5 DNA isolation

In publications **I** and **III**, viral DNA as well as DNA from EPC and BF-2 cells and from pike fry was extracted with the QiaAmp DNA mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. In publication **II**, CodV DNA was isolated from concentrated virus preparations by ultracentrifugation. Rmax DNA was extracted from growth medium collected from infected cell culture after freezing and thawing three times. Twenty microlitres of ultracentrifuged virus suspension or 200 µl of growth medium was

used in DNA isolation. The preparations were lysed with 200 µl of DNA extraction buffer containing 10 nm Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA and 2% SDS. Proteinase K was added to a final concentration of 200 µg/ml and the lysates were incubated overnight at 37 ºC. The lysates were extracted twice with phenol-chloroform, and DNA was precipitated overnight at -20 ºC with ethanol containing 0.3 M sodium acetate. The DNA was dissolved in 10-20 µl of sterile water. The DNA of BIV, EHNV, ECV, ESV, FV3, PPIV, REV 282/I02 and SERV was extracted from infected BF-2 cells with the QiaAmp DNA mini kit. The isolation of plasmid DNA (**III**, **IV**) is described in section 4.3.9.

4.2.6 RNA isolation

In publication **IV**, RNA was isolated from the ranavirus-infected and negative control cells using the RNaeasy Mini Kit (Qiagen) according to the manusfacturer's protocol. The cells were disrupted with QIAshredder spin-columns (Qiagen). On-column DNase I digestion was performed during RNA isolation (RNase-free DNase Set, Qiagen) in order to ensure the removal of genomic DNA (gDNA).

4.2.7 Reverse transcription of the isolated RNA

The concentration of the isolated RNA was measured by spectrophotometry (Eppendorf BioPhotometer, Eppendorf AG, Germany) and 200 ng of total RNA from each sample was used in cDNA synthesis in publication **IV**. In addition to RNA, the cDNA reactions contained 2.5 µM random primers (Random hexamers, Applied Biosystems, Foster City, CA, USA), 2 µl 10x PCR buffer (Applied Biosystems), 2 mM dNTPs (Applied Biosystems), 3.75 mM $MgCl_2$, 20 units of RNase inhibitor (Applied Biosystems), 100 units of MuLV Reverse Transcriptase (Applied Biosystems) and sterile water to a final volume of 20 µl. Possible gDNA contamination was monitored by including non-reverse transcriptase controls into the cDNA synthesis. The cDNA reactions were incubated at 37 ºC for 90 min and stored at -20 ºC until use.

4.2.8 Conventional PCR

Conventional PCR methods were used in publications **I**, **II**, **III** and **IV**. In publication **I**, PCR primers and conditions previously published by Hyatt et al. (2000) were used to amplify major capsid protein (MCP) gene in three overlapping fragments (Table 5). Additionally, PCR methods amplifying partial ranavirus DNA polymerase (DNApol) and neurofilament triplet H1-like protein genes (NF-H1) were developed. The primers for DNApol PCR were designed based on published sequences of FV3 (GenBank ID: AY548484) and SGIV (GenBank ID: AY521625). The expected amplicon size was 560 bp in length. The NF-H1 PCR primers were deduced from the FV3 sequence (GenBank ID: AY548484) and the expected size of the PCR product was 639 bp. The reaction

conditions for DNApol and NF-H1 PCR were the following: 35 cycles of 95ºC for 1 min, 50 ºC (for DNA pol) or 55 ºC (for NF-H1) for 1 min and 72 ºC for 1 min. The reactions for both DNApol and NF-H1 PCR contained 0.5 µM of each primer, 160 µM of each nucleotide (dATP, dTTP, dGTP, dCTP), 10x PCR buffer (150 mM Tris-HCL, 500 nM KCl, pH 8.0), 1 mM $MgCl_2$ and 2 U of *Taq* polymerase (Applied Biosystems).

In addition to the DNApol primers described in publication **I**, new PCR primers were used to amplify segments of the MCP gene of ranaviruses studied in publication **II**. Two primer pairs (MCP-AF & MCP-AR and MCP-BF & MCP-BR) were designed based on the published FV3 sequence (GenBank ID: U36913). The PCR product size was 626 bp for primer pair MCP-AF & MCP-AR and 548 bp for primers MCP-BF & MCP-BR. These two primer pairs were used with primers MCP-5 & MCP-6R published by Hyatt et al. (2000) to amplify the complete MCP gene in three segments. Additionally, PCR primers amplifying partial gene fragments of ranavirus RNase III, ribonuclease reductase alpha (RNR-α) and beta (RNR-β) genes were designed. RNase III primers were deduced from published sequences of FV3 (GenBank ID: AY548484), grouper iridovirus (GIV, GenBank ID: AY666015) and tiger frog virus (TFV, GenBank ID: AF389451). The RNR-α and RNR-β primers were designed based on the above-mentioned FV3 and TFV sequences. The expected amplification product sizes were: 717 (RNase III), 806 (RNR-α) and 646 bp (RNR-β). The cycling conditions for the MCP primers developed in study **II** were the same as with the NF-H1 primers in publication **I**. For RNR-α and RNR-β primers, the PCR conditions were 25 cycles of 95 ºC for 1 min, 50ºC for 2 min, 72ºC for 2 min. Apart from the primers, the content of the MCP, RNR-α and RNR-β PCR reactions was identical to the DNApol and NF-H1 reactions described in publication **I**.

In publication **III**, positive control plasmids containing either partial ranavirus DNApol or EPC cell glucokinase (GK) gene sequence were constructed. The primers DNApol-stF and DNApol-stR were designed based on the FV3 sequence (GenBank ID: AY548484) to amplify the partial (600 bp) viral DNApol gene. The PCR primers for the GK gene were deduced from GK mRNA sequences of common carp (GenBank ID: AF053332) and rainbow trout (GenBank ID: AF053331) and the PCR product obtained was 609 bp in length. Both DNApol and GK PCR products were inserted into plasmid vectors as described in section 4.3.9.

To determine the origin of the EPC cells in use, three separate DNA extrations from the cell culture were performed using the QIAamp DNA Mini Kit (Qiagen) in publication **IV**. The DNA was used as a template in PCR, where partial cytochrome oxidase subunit I (COx1) gene was amplified using the primers and conditions published by Winton et al. (2010). The PCR fragments from three different amplification reactions were inserted into a vector and sequenced as described in sections 4.3.9 and 4.3.12.

To obtain sequence data on the EPC cells used, several PCR primer pairs were used to amplify fragments of the following genes (**IV**): β2-microglobulin (β2M), tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 10 (IL-10), transforming growth

factor β (TGF- β) and small ribosomal subunit (40s) rRNA. All primers were designed based on sequences available in GenBank except for the reverse TNF-α primer that had been published by Stolte et al. (2008).

Table 5. *PCR primers used in this study. Primers originally published by (a) Hyatt et al. (2000), (b) Stolte et al. (2008) and (c) Winton et al. (2010).Y = C or T, K = G or T, S = C or G, R = A or G, V = A or C or G.*

Target gene	Primer name	1) 5'-forward primer-3' 2) 5'-reverse primer-3'	Publication
MCP	MCP-1	1) CACCGTGTATCTTATAATAAAAAGGAAATG	a, **I**
	MCP-2R	2) GGCTCCGTCCTGGCCTGTG	a , **I**
	MCP-3	1) GAGGCCAAGCGCACAGGCTAC	a , **I**
	MCP-4R	2) TTGGAGCCGACGGAAGGGTG	a , **I**
	MCP-5	1) CGCAGTCAAGGCCTTGATGT	a, **I, II**
	MCP-6R	2) AAAGACCCGTTTTGCAGCAAAC	a, **I, II**
	MCP-AF	1) CCTCCAAAGAGAGCGATATGC	**II**
	MCP-AR	2) AAGAATGGGAGGGGAAGA	**II**
	MCP-BF	1) ACCAGCGATCTCATCAAC	**II**
	MCP-BR	2) AGCGCTGGCTCCAGGACCGT	**II**
DNApol	DNApol-F	1) GTGTAYCAGTGGTTTTGCGAC	**I, II**
	DNApol-R	2) TCGTCTCCGGGYCTGTCTTT	**I, II**
	DNApol-stF	1) AAAGACTCCGCACAGCGTCG	**III**
	DNApol-stR	2) GGTCCCTGAGGCTGGTGAGC	**III**
NF-H1	NF-H1-F	1) CCAAAGACCAAAGACCAG	**I**
	NF-H1-F	2) GTTGGTCTTTGGTCTCGCTC	**I**
RNase III	RNase III-F	1) GAGGCKCTGGAGATYGTGGGSGA	**II**
	RNase III-R	2) CCCRCTRCCCTCVACRAC	**II**
RNR-α	RNR-AF	1) CTGCCCATCTCKTGCTTTCT	**II**
	RNR-AR	2) CTGGCCCASCCCATKGCGCCCA	**II**
RNR-β	RNR-BF	1) AGGTGTRCCRGGGYCGTA	**II**
	RNR-BR	2) GACGCTCCAYTCGACCACTT	**II**
GK	GK-stF	1) GTTCAAGGCGTCTGGAGCAGAG	**III**
	GK-R1	2) CCCACTCTGTGTTCACACACATCC	**III**
β2M	-	1) GCTGTACRTCACTGTACARGGG	**IV**
	-	2) TTACATGTTGGGCTCCCAA	**IV**
TNF-α	-	1) TTACCGCWGGTGATGGTGTC	**IV**
	-	2) CCTTGGAAGTGACATTTGCTTTT	b, **IV**
IL-1β	-	1) CAGCCTGTGTGYYTGGGAAT	**IV**
	-	2) TGCCGGTYTCYTTCCTGAAG	**IV**
IL-10	-	1) TGGAGTCATCCTTTCTGCTCTG	**IV**
	-	2) CATTTCMSCATATCCCGCTTGAG	**IV**
TGF- β	-	1) GGACAGATTCTCAGCAARCTACG	**IV**
	-	2) TACAGCTCCAGTCTCTGCTCTG	**IV**
40s	-	1) GAAGATGCAGAGGACCATCG	**IV**
	-	2) GAACTTCTGGAACTGCTTCTTG	**IV**
COx1	-	1) CTTTATCTAGTATTTGGTGCC	c, **IV**
	-	2) CCTGCMGGGTCAAAGAATGT	c, **IV**

4.2.9 Cloning of PCR products

For the quantitation of viral or host DNA by standard curve, positive control plasmids containing either the viral or the host target gene sequence were constructed in publication **III**. Primers DNApol-stF and DNApol-stR were used to amplify partial DNApol gene of FV3. A fragment of the GK gene of EPC cells was amplified using primers GK-stF and GK-R1. The amplified sequences were inserted into pSC-A plasmids and transfected into StrataClone SoloPAck competent cells (StrataClone PCR Cloning Kit, Agilent Technologies, La Jolla, CA, USA). The cells were grown at 37°C in Luria-Bertani broth (LB)-agar plates in the presence of ampicillin (100 µg/ml) and X-gal (5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside). White colonies were selected and grown overnight in LB medium. The plasmid DNA was purified using the QIAprep Spin Miniprep kit (Qiagen). The presence of the insert sequence was ensured by sequencing the plasmid DNA as described in section 4.3.12. The concentrations of the DNApol and GK plasmid DNA were determined by spectrophotometric analysis and ten-fold dilution series from 10 to 10^8 copies of a plasmid containing either the partial DNApol or GK gene were prepared.

In publication **IV**, the origin of the EPC cells was investigated. PCR fragments from three amplification reactions of a partial COx1 gene of EPC cells were used in three individual cloning reactions as described above. The plasmid DNA was purified from ten colonies from each cloning reaction using QIAprep Spin Miniprep kit. The sequencing of the inserts is described in section 4.3.12.

4.2.10 Quantitative real-time PCR

In study **III**, quantitative real-time PCR (qPCR) assays based on a standard curve were developed for the detection and quantitation of ranaviruses. Target genes for the assays were viral DNApol and GK of the EPC cells. Altogether, three primers for the DNApol qPCR were used; two of them were deduced from EHNV and DFV sequences produced in publication **I** (GenBank IDs: FJ374274 and FJ374281, respectively), and one of the primers (DNApol-F) was described in publication **I** (Table 6). For GK qPCR, four primers were designed based on published mRNA sequences of common carp and rainbow trout. All primer pair combinations for both target genes were tested at three annealing temperatures: 50, 55 and 60 ºC. The efficiency of the primer pairs was estimated by the slope of the regression curve obtained with ten-fold dilutions of the positive control plasmids. Melting curve analysis was used to evaluate the specificity of the primer pairs. One primer pair for each target gene was selected based on their specificity and efficiency. Each sample was run in four replicates with both DNApol and GK qPCR. The qPCR reactions contained 7.5 µl of Dynamo Flash SYBR Green qPCR Mastermix (Finnzymes, Espoo, Finland), 250 nm of each primer, 1x ROX passive reference dye, 5 µl of template DNA and sterile water to a final volume of 15 µl. The samples were run in 384-well plates with the ABI 7900HT Sequence Detection System (Applied Biosystems) using the

following PCR conditions: 7 min incubation at 95 ºC, 40 cycles of 10 s at 95 ºC and 30 s at 60 ºC for DNApol or 50 ºC for GK, and 1 min at 60 ºC. The specificity of DNApol qPCR was tested with the DNA of two non-ranaviruses: koi herpesvirus (KHV) and red sea bream iridovirus (RSIV) in addition to ten different ranavirus isolates. The sensitivity and reproducibility of the assays was tested with ten-fold dilutions of the positive control plasmid DNA. The quantity of the target DNA was determined using a standard curve method. The same amount of DNA from each sample was tested both with DNApol and GK qPCR and the copy number was determined from a standard curve generated with ten-fold dilutions of the positive control plasmids. The viral DNApol copy number was divided by the fish cell GK copy number of each sample in order to obtain a viral quantity value, virions per host cell.

In publication **IV**, qPCR assays were developed in order to examine immune gene expression in ranavirus-infected EPC cells. Primers and probes targeting β2-microglobulin (β2M), tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 10 (IL-10), transforming growth factor β (TGF-β) and small ribosomal subunit (40s) rRNA genes were designed based on the sequence data obtained in the study and by using Primer Express 3.0 software (Applied Biosystems). The optimal PCR conditions were determined with two concentrations of the forward and reverse primers (100 and 300 nM) and probes (100 and 250 nM) for all target genes. The concentrations producing the lowest cycle threshold (Ct) values in PCR were chosen for the qPCR analysis. The PCR specificity for each primer pair was determined by gel electrophoresis. PCR efficiency for each primer pair was determined using standard curves generated with two-fold dilutions of the standard cDNA template that was prepared from the RNA of EPC cells stimulated with LPS, CpG-ODN and poly I:C. The qPCR reactions contained 5 µl TaqMan Universal PCR Master Mix with AMPerase UNG (Applied Biosystems), 300 nM forward and reverse primer, 100 nM probe, 3 µl of cDNA and water up to a final volume of 10 µl. A standard cDNA template was included into each plate in order to monitor the reproducibility of the qPCR assays. All samples were run in triplicate with the StepOnePlus Real-Time PCR System (Applied Biosystems). The cycling conditions were the following: initial incubation steps at 50 ºC for 2 min (AmpErase UNG activation), and at 95 ºC for 10 min (hot-start DNA polymerase activation), followed by 40 cycles of 15 s at 95 ºC and 1 min at 60 ºC. Raw data were analysed with StepOne Software v2.0 (Applied Biosystems). The Ct values were determined using a constant threshold value 0.03 for all genes and samples. Relative quantitation of β2M, TNF- α, IL-1β, IL-10 and TGF-β expression was calculated by the comparative Ct method (Livak & Schmittgen 2001). The relative quantitation value of the target, normalised to an endogenous control and relative to the calibrator, is expressed as $2^{-\Delta\Delta Ct}$. In this study, ΔCt = Ct of the target gene (β2M, TNF- α, IL-1β, IL-10 or TGF-β) - Ct of the endogenous control gene (40s). ΔΔCt = ΔCt of the sample – ΔCt of the calibrator sample. The fold change in the expression values was calculated by dividing the relative gene expression value of the treated sample (infected with ranavirus or treated with immunostimulant) by the relative gene expression value of the negative control.

Table 6. *qPCR primers and probes used in the study.*

Target gene	Primer name	1) 5'-forward primer-3' 2) 5'-reverse primer-3' 3) 5'-(FAM)-probe-(BHQ1)-3'	Publication
DNApol	DNApol-F	1) GTGTAYCAGTGGTTTTGCGAC	**III**
	DNApol-R1	2) CGTAAAASCCKGRCCTGAARCC	
	DNApol-R2	2) GAARCCMGTCACCCTMACGCAGAC	
GK	GK-F1	1) GTAAYGCKTGYTACATGGAGGAG	**III**
	GK-F2	1) GCKTGYTACATGGAGGAG	
	GK-R1	2) CCCACTCTGTGTTCACACACATCC	
	GK-R2	2) CTGTGTTCACACACATCC	
β2M	-	1) CCCTCCTGATATCTCCATTGAACT	**IV**
	-	2) CTGCCAGCCCTTTTCGAA	
	-	3) TTATCCCTAACTCCCAGCAGACTGACCTGG	
TNF-α	-	1) CAAGCAATTGGCGAGTGTGT	**IV**
	-	2) CAGTTCCACTTTCCTGATTACTCTGA	
	-	3) TGCTGCTGTTTGCTTCACGCTCAAC	
IL-1β	-	1) AGACCAATCTCTACCTCGCTTGTAC	**IV**
	-	2) TTAATGGTGTTTAATGTTTCACTGATCTC	
	-	3) ACGATGCTTCCCCCCACCTGG	
IL-10	-	1) GATGTCACGTCATGGACGAGAT	**IV**
	-	2) GGACTGGAAGTGGTTCTTCTGTACA	
	-	3) CTGCGCTTCTACTTGGAAACCATTCTGC	
TGF-β	-	1) TGTATAACAGCACTGTCGAGCTAAGC	**IV**
	-	2) TCCCTTCTCATTAGGATCTTCTACATC	
	-	3) AGCAGGCGGCAGATCCTGTACACC	
40s	-	1) TTTTGAGAAGAGGCATAAGAACATGT	**IV**
	-	2) GTAACGATGTCACCAACAGTCACA	
	-	3) TGTCCACCTCTCTCCATGCTTCAGGG	

4.2.11 Restriction enzyme analysis

Restriction enzyme analysis (REA) of PCR-amplified fragments of viral DNApol and NF-H1 genes was performed in order to differentiate the ranavirus isolates in publication **I**. The DNApol PCR products were digested with restriction enzyme *Bpm*I and those of NF-H1 with *Alu*I, *Hae*III and *Afl*III (New England Biolabs). The reaction mixtures consisted of 20 to 30 µl of PCR product, 2.5 to 5 U of the respective restriction enzyme, 4 µl of 10x restriction enzyme buffer, and sterile water to a final volume of 40 µl. Reaction mixtures containing *Bpm*I and *Afl*III were supplemented with 0.5 µl of bovine serum albumin (10 mg/ml). The reactions were incubated for 1 to 4 h at 37ºC and analysed after gel electrophoresis.

4.2.12 Sequencing

The nucleotide sequences of PCR products (**I**, **II**, **III** and **IV**) or plasmid DNA (**III** and **IV**) were determined by dye-terminator sequencing. The PCR products were purified using MinElute PCR purification columns (Qiagen) and sequenced using the Big Dye Terminator v1.1 Cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. In publications **III** and **IV**, the plasmid DNA was sequenced with the Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems). Unincorporated dye terminators were removed by column purification (DyeEx 2.0 spin kit, Qiagen), and the sequencing reactions were run on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

4.2.13 Sequence analyses

The raw sequence data were analysed using Sequencing Analysis Software 5.1 (Applied Biosystems). In publication **II**, the individual gene sequences of each virus isolate were assembled into one continuous sequence using SeqMan Pro v.8.0.2 from the DNASTAR Lasergene 8 application package (DNASTAR Inc.). Multiple sequence alignments were performed with ClustalX 1.81 (Thompson et al. 1997) (**I**, **II**). Sequence pair percent identity values were calculated using the MegAlign program (DNASTAR Lasergene 8) in publications **I** and **II**. The nucleotide sequence data in publications **I** and **II** were subjected to maximum parsimony phylogenetic analysis performed with Mega 4.1 software (Tamura et al. 2007). The reliability of the obtained phylogeny was tested by bootstrapping.

4.2.14 Indirect fluorescent antibody test (IFAT)

In publication **II**, a 1:1000 dilution of each of the ranavirus isolates studied (BIV, CodV, ECV(It), ECV(Fr), EHNV, ESV, FV3, PPIV, REV 282/I02, Rmax, and SERV) was added to glass cover slip cultures of EPC cells and incubated at 15 ºC for 72 h. Cover slips were then rinsed and fixed in 80% acetone prior to immunofluorescence staining as described previously (Jørgensen et al. 1989). Primary antibodies (rabbit sera) against different ranavirus isolates (anti-BIV, -CodV, -ECV(Fr), -ECV(It), -EHNV, -ESV, -PPIV and -Rmax) were tested for reactivity to homologous and heterologous isolates at 1:800 and incubated for 30 min at 37 ºC. In addition, a monoclonal antibody against RSIV was tested at a 1:10 dilution, as were antisera against infectious pancreatic necrosis virus (IPNV) serotypes Sp and Ab (Jørgensen & Grauballe 1971), and lake trout rhabdovirus (LTR, Koski et al. 1992) at a dilution of 1:1000. Rhodamine- or fluorescein-conjugated swine antibodies to rabbit immunoglobulin (R015 and F0205, respectively, Dako, Copenhagen, Denmark) were applied as secondary antisera at a dilution of 1:100 and 1:40, respectively, for 30 min at 37 ºC.

4.2.15 Detection of apoptosis

In publication **IV**, the ability of EHNV, FV3, ECV and DFV to induce apoptosis in EPC cells was investigated. EPC cells were plated onto glass cover slips (~800 000 cell/cover slip) and infected with the four isolates with an MOI of 2.5. At 6, 24 and 48 h p.i., three staining reactions for each viral isolate were performed. The cells were stained with Hoechst 33258 (HO, 5µg/ml, Sigma-Aldrich) and propidium iodide (PI, 5µg/ml, Sigma-Aldrich) for 30 min at 37ºC. Negative controls were included in each staining. After the staining, the cells were rinsed three times with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and rinsed three times with PBS. The cover slips were mounted on slides and viewed under a Leica DM 400B (Wetzlar, Germany) fluorescence microscope. The proportion of apoptotic cells was estimated by counting the cells from 10 randomly chosen areas in each cover slip. Mean values and the standard error of the mean were calculated for each viral treatment group and time point.

Both HO and PI are stains that bind to DNA. HO is able to penetrate all cell membranes and stains DNA blue. PI enters necrotic and late-phase apoptotic cells that have lost their membrane integrity, and induces red staining of DNA. Apoptotic cells can be detected by their fragmented nuclei with blue staining (HO). In necrotic cells, staining with both HO and PI appears and the cells exhibit white fluorescence.

4.2.16 Statistical analyses

In publication **III**, the pairwise comparisons of the viral quantities in EPC and BF-2 cells and the EHNV quantities between fish from different experimental tanks were carried out by using the non-parametric Mann-Whitney U-test. The correlation analyses in studies **III** and **IV** were performed with Spearman's rank correlation test. The groupwise comparisons in study **IV** were conducted with one-way analysis of variance (ANOVA) and Dunnett's test or with non-parametric Kruskall-Wallis and Dunn's tests. In Dunnett's and Dunn's tests, p-values were adjusted for multiple comparisons. All analyses were performed with Graph Pad Prism software (Graph Pad Software Inc.). P-values less than 0.05 were considered significant.

5 Results

5.1 PCR and DNA sequence analyses of different ranaviruses (I, II)

In publications **I** and **II**, selected genes of 13 different viral isolates were investigated. Complete or partial fragments of six viral genes were amplified with PCR and sequenced. Novel sequences were submitted to GenBank. The target genes and the GenBank accession numbers are presented in Table 7.

Table 7. *Viral genes amplified by PCR in studies I and II. The number of publication and the GenBank ID for novel sequences are given for each gene. ND, not done.*

	Target gene					
Isolate	**MCP**	**DNApol**	**NF-H1**	**RNase III**	**RNR-α**	**RNR-β**
BIV	**I** FJ358613	**I** FJ374280	**I** FJ391462	**II** GU391273	**II** GU391286	**II** GU391264
CodV	**II** GU391284	**II** GU391282	ND	**II** GU391274	**II** GU391287	**II** GU391265
DFV	ND	**I** FJ374281	ND	ND	ND	ND
ECV	**I** FJ358608	**I** FJ374277	**I** FJ391464	**II** GU391275	**II** GU391288	**II** GU391266
EHNV	**I**	**I** FJ374274	**I** FJ391467	**II** GU391276	**II** GU391289	**II** GU391267
ESV	**I** FJ358609	**I** FJ374278	**I** FJ391465	**II** GU391277	**II** GU391290	**II** GU391268
GV6	ND	**I** FJ374282	ND	ND	ND	ND
FV3	**I** FJ459783	**I**	**I**	**II**	**II**	**II**
PPIV	**I** FJ358610	**I** FJ374276	**I** FJ391467	**II** GU391278	**II** GU391292	**II** GU391269
REV 282/I02	**I** FJ358611	**I** FJ37475	**I** FJ391461	**II** GU391280	**II** GU391293	**II** GU391271
Rmax	**II** GU391285	**II** GU391283	ND	**II** GU391279	**II** GU391291	**II** GU391270
SERV	**I** FJ358612	**I** FJ374279	**I** FJ391463	**II** GU391281	**II** GU391294	**II** GU391272
SGIV	ND	**I**	ND	ND	ND	ND

The complete MCP and partial RNAse III, RNR-α and RNR-β gene sequences were obtained from ten isolates: BIV, CodV, EHNV, FV3, ECV, ESV, PPIV, REV 282/I02, Rmax and SERV. In all isolates, the MCP gene was 1392 bp in length and corresponded in size to the published data on EHNV, BIV (Marsh et al. 2002) and FV3 (Mao et al. 1996, Tan et al. 2004). One nucleotide difference was detected in the MCP sequence of both BIV and FV3 compared to the published data (BIV: GenBak ID AY187046, FV3: GenBank ID AY548484). There were no differences in the sizes of RNAse III, RNR-α or RNR-β PCR products between the ten isolates. The RNAse III, RNR-α and RNR-β sequences of FV3 were identical to the published FV3 genome sequence (GenBank ID AY548484).

A partial NF-H1 gene sequence was successfully obtained from eight of the isolates studied: BIV, EHNV, FV3, ECV, ESV, PPIV, REV 282/I02 and SERV. Based on the agarose gel electrophoresis and sequencing results, the sizes of the NF-H1 PCR products varied between isolates: 714 (BIV), 597 (EHNV), 864 (ECV), 759 (ESV), 639 (FV3), 612 (PPIV), 579 (REV 282/I02) and 588 bp (SERV). Some of the obtained NF-H1 amplicons contained repetitive sequences. In ECV, the amplified NF-H1 region included 18 consecutive repetitions of 15 nucleotides (5′ CCAGCGAGAAAGTCT 3′, corresponding to amino acids PARKS), whereas in ESV, 11 similar repetitions were found. Repetitive sequences were observed also in FV3 and BIV. Excluding the repetitive regions, ECV and ESV were identical with each other and SERV was the most divergent of the isolates studied. The NF-H1 sequence identity between the eight isolates varied from 77.2 to 100%.

A partial DNApol gene sequence was obtained from all thirteen isolates. The size of the PCR product varied between isolates: 554 bp in DFV and GV6, and 560 bp in BIV, CodV, EHNV, FV3, ECV, ESV, PPIV, REV 282/I02, Rmax, SERV and SGIV. The obtained DNApol sequences of FV3 and SGIV were identical to the published sequences of these isolates (Song et al. 2004: GenBank ID NC_006549, Tan et al. 2004: GenBank ID AY548484).

Based on the gene regions studied, there was little genetic variance between BIV, CodV, EHNV, FV3, ECV, ESV, PPIV, REV 282/I02, Rmax and SERV. Excluding the NF-H1 sequences, the nt sequence identity of this group of viruses was more than 95%. With the exception of the repetitive sequences in the NF-H1 region, ECV and ESV were highly similar to each other with only one nucleotide difference in RNase III sequence. DFV and GV6 were also identical based on the DNApol sequences. Nevertheless, all isolates, with the exception of GV6 and DFV, could be differentiated based on the sequence data obtained. The most divergent isolates were DFV, GV6 and SGIV, with an nt sequence identity between 65.9 and 70.6 % in DNApol compared to the other isolates. Based on the obtained DNApol sequences of GV6 and DFV6 and the published sequences of SGIV, these three viruses also had a lower GC content (~49 %) in the studied gene sequences compared to the other isolates. In the maximum parsimony analysis, GV6 and DFV as well as SGIV clearly clustered apart from the rest of the ranaviruses studied (Fig.3).

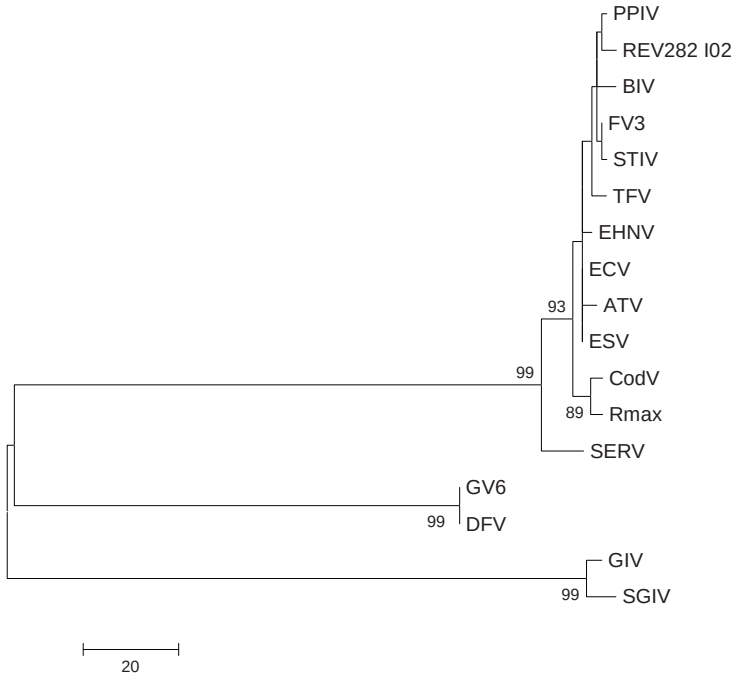


Figure 3. *Maximum parsimony analysis based on partial DNApol gene sequences of ranaviruses. Sequences of PPIV, REV 282/I02, BIV, EHNV, ECV, ESV, CodV, Rmax, SERV, GV6 and DFV were obtained in studies* ***I*** *and* ***II****. GenBank ID numbers for the previously published sequences: FV3 (AY548484), STIV (NC_012637), tiger frog virus (TFV, AF389451), Ambystoma tigrinum virus (ATV, NC_005832), GIV (AY666015) and SGIV (NC_006549).*

5.2 Differentiation of ranaviruses by REA (I)

GV6 and DFV had an identical DNApol sequence and they were distinguished as a group from the other isolates by *Bpm*I digestion of the DNApol PCR products. With the same enzyme, a unique restriction pattern was obtained for SERV and SGIV. Further differentiation of BIV, EHNV, ECV, ESV, REV 282/I02 and PPIV was accomplished with REA of NF-H1 PCR products using *Alu*I, *Hae*III and *Afl*III. The *Alu*I digestion produced distinct patterns for BIV, ECV, ESV, SERV and REV 282/I02. Using *Hae*III, EHNV, FV3, BIV, ECV and SERV could be differentiated from the other isolates. With *Afl*III, clearly dissimilar patterns for EHNV, ECV and ESV were obtained. Table 8 summarizes the restriction enzyme reactions needed to distinguish each viral isolate.

Table 8. *Differentiation of ranaviruses by REA of DNApol and NF-H1 PCR products. X: a distinct restriction pattern was obtained with the respective enzyme. Bpm*I *digestion of DNApol PCR products of GV6 and DFV produced an identical pattern. To differentiate PPIV, both Alu*I *and Hae*III *digestion of NF-H1 PCR products is needed.(-): isolate could not be differentiated with the respective enzyme.*

	DNApol	NF-H1		
Isolate	***Bpm*I**	***Alu*I**	***Hae*III**	***Afl*III**
EHNV	-	-	X	X
FV3	-	-	X	-
BIV	-	X	X	-
ECV	-	X	X	X
ESV	-	X	X	X
SERV	X	X	X	-
REV 282/I02	-	X	-	-
PPIV	-	X		-
DFV/GV6	X	-	-	-
SGIV	X	-	-	-

5.3 Detection of ranaviruses using IFAT (II)

Polyclonal antisera against ranaviruses reacted strongly in IFAT with the ranavirus isolates studied. Positive staining was detected in the cytoplasms of the infected EPC cells for all combinations isolates and ranavirus antisera. Due to the complete cross-reaction with all of the ranavirus antisera tested, differentiation of the ranavirus isolates was not possible. The ranavirus antisera produced no staining when tested with non-ranaviruses IPNV or LTR. Furthermore, no staining was observed when the monoclonal antiserum against non-ranavirus RSIV was tested with the ranavirus isolates, IPNV or LTR.

5.4 Challenge trials with rainbow trout (II)

In the rainbow trout challenged with BIV, CodV, EHNV, ECV (Fr), ECV (It), ESV, FV3, PPIV and Rmax, mortality was low (max 2/50 fish per tank) and did not exceed the mortality recorded in the placebo treatments. In the positive control tanks with fish infected with viral haemorrhagic septicaemia virus (VHSV), the accumulated mortality reached 70% by day 28, and VHSV was isolated from the dead fish. Of the ranavirus-challenged fish collected on day 7, three positive viral isolations were made from individual fish bath treated with EHNV, FV3 and ESV. Of the fish collected on day 28, no viruses were isolated. In the ESV and EHNV challenges, virus was isolated from the dead fish in the third week of the experiment. The viruses isolated from challenged fish were

confirmed as ranaviruses in IFAT using anti-EHNV antisera. No pathological changes were detected in the histopathological examination of 50 fish (10 from 5 tanks).

5.5 Quantitation of ranaviruses (III)

The developed DNApol qPCR amplified the expected 93 bp fragment from all ten ranavirus isolates studied (BIV, DFV, EHNV, ECV, FV3, GV6, PPIV, REV 282/I02 and SERV). The assay was specific; no amplification was detected in PCR reactions where gDNA of non-ranaviruses red sea bream iridovirus (RSIV) or koi herpesvirus (KHV) was used as a template. The GK qPCR assay amplified a partial GK gene (79 bp) from EPC and BF-2 cells as well as from pike tissue. The sensitivity and reproducibility of the qPCR assays was examined using serial ten-fold dilutions of the positive control plasmids. The dilutions were run with both assays and standard curves were generated based on the Ct values obtained. The linear range for the DNApol qPCR was between 10 and 10^8 copies, and the coefficient of determination (R^2) for the standard curve was 0.997. For the GK qPCR the linear range was between 100 and 10^8 copies and the R^2 was 0.992. Both assays were highly reproducible, the inter-assay coefficient of variation (CV) of the mean Ct values varying between 0.36 and 2.02 for the DNApol qPCR and between 0.38 and 1.67 for the GK qPCR.

At the beginning of the study period, EHNV and ECV quantities were higher in EPC cells, but in the end of the experiment the highest viral quantities were measured from BF-2 cells for both isolates ($p < 0.01$). The quantities of FV3 and DFV were higher in EPC cells throughout the observation period ($p < 0.01$), except for DFV at 72 h p.i., when the difference in viral quantities between the two cell lines was not significant. In general, the detected quantities of EHNV and FV3 were higher than those of ECV and DFV in both cell lines at the end of the experiment. Of all four isolates, the highest viral quantity in the EPC cells was measured with FV3 (mean value 617 virions/cell) at 72 h p.i. and in the BF-2 cells with EHNV (358 virions/cell) at 48 h p.i. (Fig. 4). EHNV was detected in all pike tissue samples studied, except for the negative controls. At 12 ºC, the highest viral quantity was detected 7 d p.i., whereas at 22 ºC, the highest quantity was measured 3 d p.i. There was no correlation between the viral quantities and the number fish in the pooled samples.

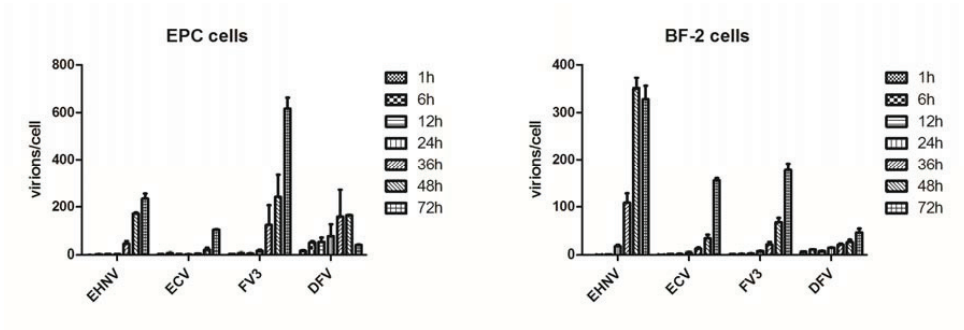


Figure 4. *The viral quantities (virions/cell) of four ranavirus isolates measured at seven time points p.i. in EPC and BF-2 cells.*

5.6 Ranavirus-induced immune response in fish epithelial cells (IV)

5.6.1 The origin of EPC cells

EPC cells are well suited to propagating ranaviruses (Ariel et al. 2009b, OIE 2009, 2012), and were therefore selected as a representative fish epithelial cell line to examine the initial host immune response to ranavirus infection. Due to the recent report on the contamination of the EPC cell line (Winton et al. 2010), the origin of the EPC cells used here was investigated. A partial COx1 gene of EPC cells was amplified and cloned into plasmid vectors. Inserts of thirty colonies from three different cloning reactions were sequenced. All sequences obtained were identical to the published fathead minnow COx1 sequence (GenBank ID: EU525089). At the time of the study, no published sequences for fathead minnow were available on the immune genes under investigation. In order to design primers and probes for mRNA expression measurements, partial β2M, TNF-α, IL-1β, IL-10, TGF-β and 40s rRNA genes of EPC cells were sequenced. All sequences obtained, except for IL-1β, were submitted to GenBank with the following ID numbers: JN412132 (β2M), JN412133 (TNF-α), JN412134 (IL-10), JN412135 (TGF-β) and JN412136 (40s). The IL-1β sequence was less than 200 bp in length and was therefore unacceptable for the GenBank database. The IL-1β sequence is presented in Figure 1 in publication **IV**.

The sequences were aligned and analysed with published sequences of carp. The differences between fathead minnow (EPC cells) and carp nucleotide (nt) and amino acid (aa) sequences were the following: 48 nt and 12 aa differences in β2M, 26 nt and 11 aa differences in TNF-α, 28 nt and 10 aa differences in IL-1β, 39 nt and 9 aa differences in IL-10, 69 nt and 29 aa differences in TGF-β and 9 nt differencesand 1 aa difference in 40s. The GenBank IDs of the carp sequences used in the analyses were L05536 for β2M,

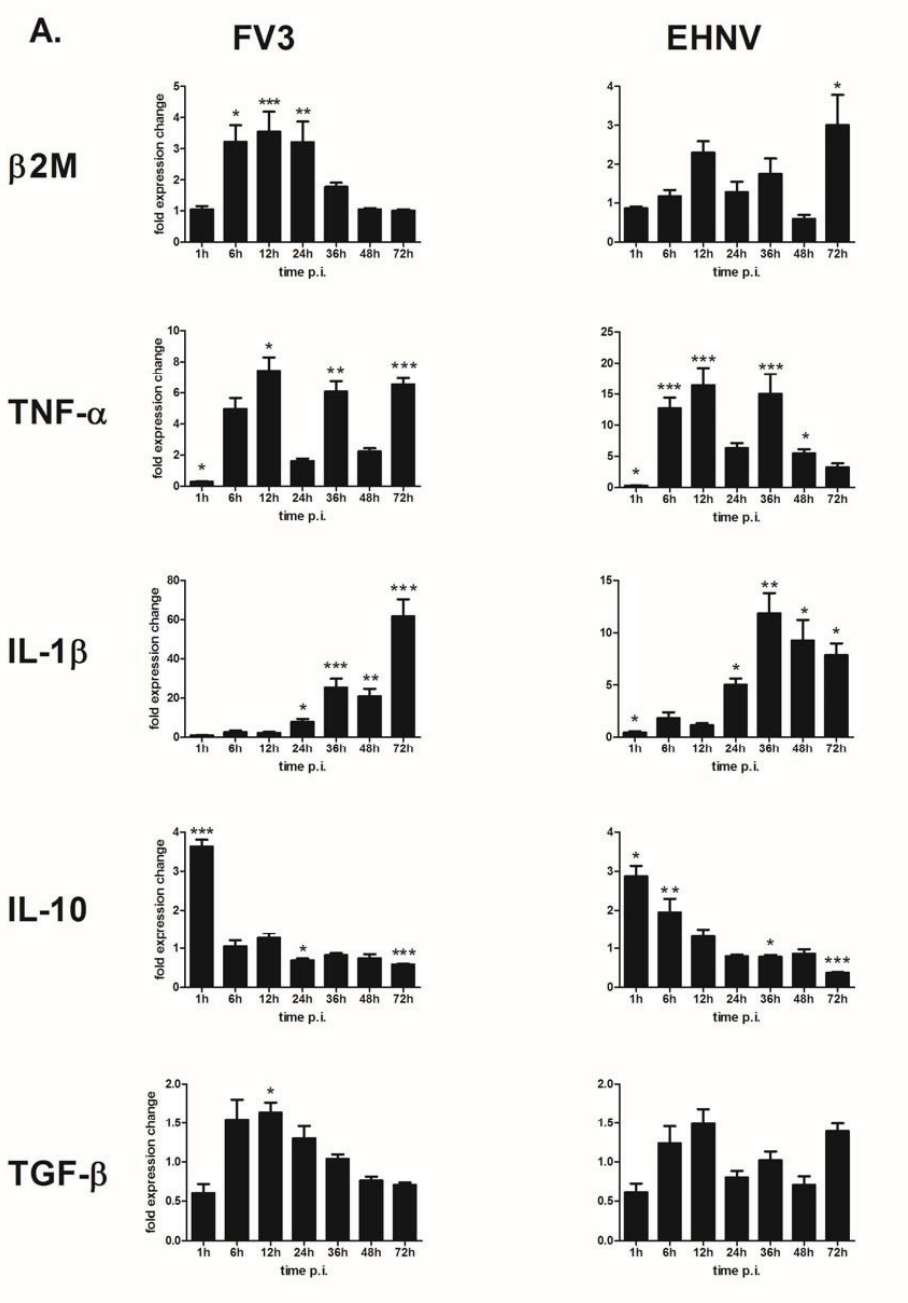
AJ311800 for TNF-α, AJ245635 for IL-1β, AB110780 for IL-10, AF136947 for TGF-β and AB012087 for 40s rRNA.

5.6.2 mRNA expression measurements of the host immune response genes

The efficiency of qPCR assays targeting β2M, TNF- α, IL-1β, IL-10, TGF-β and 40s was determined using the standard curves generated with two-fold dilutions of the standard cDNA template. The six qPCR assays produced a strong linear fit with the cDNA template dilutions and the PCR efficiency was ≥ 92% for all assays. The inter-assay CVs of the mean Ct values were ≤ 2.6 for all genes, indicating that the assays were highly reproducible.

Ranaviruses induced changes in the expression of the immune genes studied. The most notable increases were detected in the expression of pro-inflammatory cytokines TNF-α and IL-1β. In all viral treatment groups, TNF-α expression was lower than in the negative control at 1 h p.i., but rapidly increased at the later time points. The greatest increase was detected at 12 h p.i. in EHNV-infected cells (16.4-fold, Fig. 5). For IL-1β, the expression increased somewhat slower: a more than two-fold increase was detected 36 h p.i. in all viral treatments. FV3 induced the most substantial increase of all ranaviruses: 61.6-fold at 72 h p.i. A more than two-fold increase in β2M expression was detected in all viral treatment groups at 12 h p.i. The greatest up-regulation was detected at 36 h p.i. in ECV-infected cells (4.2-fold). IL-10 expression at 1 h p.i. was higher in FV3, EHNV, and DFV-infected cells than in the negative control. Later, the expression in the negative control cells increased more than in the ranavirus-infected cells, and by the end of the study period, the IL-10 expression was lower in all viral treatments compared to the negative control ($p < 0.001$). A modest, but statistically significant increase in TGF-β expression was detected at two time points in cells infected with DFV and ECV. Infection with FV3 or EHNV did not induce a notable increase in TGF-β expression during the study period.

In addition to ranaviruses, the other microbial stimulants evoked measurable changes in the immune gene expression in EPC cells. The most notable increase in TNF-α expression was detected in LPS-stimulated cells at 6 h p.i. (~362-fold). Additionally, LPS induced up-regulation in IL-1β expression. CpG-ODN induced a greater than two-fold increase in β2M, IL-1β and TNF-α expression, whereas poly I:C elicited a statistically significant increase in β2M, IL-10 and TGF-β expression ($p < 0.05$), and a more than two-fold up-regulation of IL-1β and TNF-α.


A.
FV3
EHNV
β2M
TNF-α
IL-1β
IL-10
TGF-β
fold expression change
time p.i.
1h
6h
12h
24h
36h
48h
72h

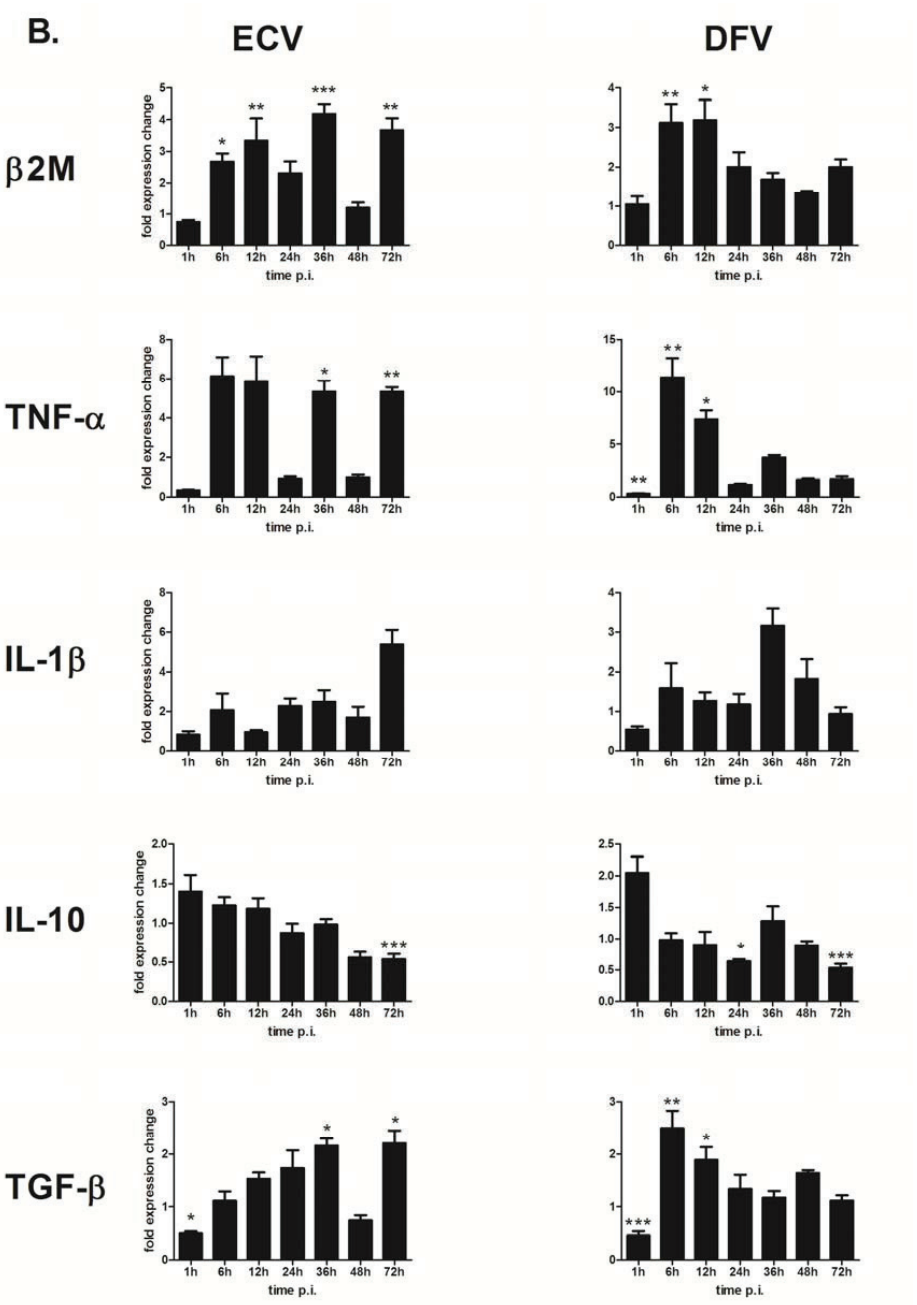

B.
ECV
DFV
β2M
TNF-α
IL-1β
IL-10
TGF-β
fold expression change
time p.i.
1h
6h
12h
24h
36h
48h
72h

Figure 5. *Variation in the expression fold change values of five immune response genes in four ranavirus treatment groups: (A) FV3 and EHNV, (B) ECV and DFV. Fold change units were calculated by dividing the relative gene expression of ranavirus-infected samples by the relative gene expression of the negative control. Error bars represent the standard error of the mean. Statistically significant differences between the treated sample and negative control are marked with asterisks: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.*

5.6.3 Viral load and immune response gene expression

Viral quantities of the samples studied in publication **IV** were determined in publication **III**. In study **IV**, the viral quantities were reanalysed to obtain a relative viral load value by dividing the viral quantity by the initial infective dose (MOI 2.5). The relative values obtained were used to study the correlation between the immune expression levels and the viral load. The relative viral load correlated significantly with IL-1β expression in FV3, EHNV and DFV-infected EPC cells ($p < 0.05$). Additionally, IL-10 expression correlated with the viral load of one of the isolates, DFV ($p = 0.034$). The expression levels of the other immune genes did not correlate with the viral loads.

5.6.4 Apoptosis

Apoptotic changes, such as chromatin condensation and multiple apoptotic bodies (Fig. 6), were observed in each of the four ranavirus treatments. In negative control cells, no changes were observed. With all ranavirus isolates, the proportion of apoptotic EPC cells was relatively low, being less than 5% at the three time points studied. The highest percentage of apoptotic cells were detected at 48 h p.i. in cells infected with FV3 (4.56 ± 0.24%), whereas the lowest percentage was detected at 6 h p.i. in ECV-infected cells (1.04 ± 0.08%). Necrotic cells were rare in all viral treatments.

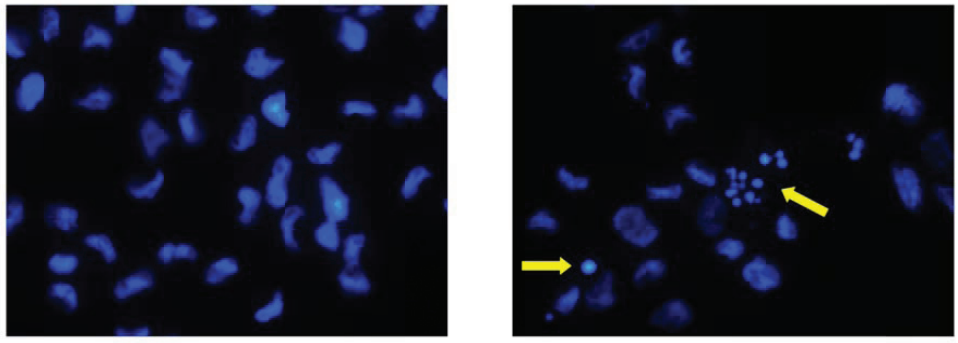


Figure 6. *Induction of apoptosis by ranaviruses in EPC cells. Negative control cells on the left, ranavirus-infected cells on the right. Apoptotic bodies are indicated with arrows. The pictures were taken at 400x magnification.*

6 Discussion

6.1 Genetic diversity within the genus *Ranavirus*

In studies **I** and **II**, sequence data from several known and previously less well characterized ranavirus isolates were obtained. The small differences in the FV3 and BIV MCP sequences compared to the published data could be explained by mutation occurring in the viral genome during propagation in cell cultures. Additionally, the original viral isolates could have contained different genetic variants, which after selection in cell culture could result in differing subpopulations. Genetically variable subpopulations could also be the reason why, contrary to results published by Hyatt et al. (2000), the amplification of MCP was not successful with DFV or GV6. A recent publication, including the same isolates of DFV and GV6 that were studied here, reported the complete and identical MCP sequences of these two viruses and confirmed that the isolates indeed differed in sequence compared to the ones studied by Hyatt and colleagues (Ohlemeyer et al. 2011). In the previous ICTV report on virus taxonomy, DFV and GV6 were categorised as isolates of the species *Santee Cooper ranavirus* (Chinchar et al. 2005), whereas in the most recent report, neither of the isolates were included in the list of members of the genus *Ranavirus* (Jancovich et al. 2011).

Based on the sequence analyses presented here and in previous studies, most of the ranavirus isolates, with the exception of Santee Cooper ranaviruses (GV6, DFV, LBMV) and grouper iridoviruses (SGIV, GIV), are genetically closely related (Mao et al. 1997, Hyatt et al. 2000, Marsh et al. 2002). The DNApol sequences of DFV, GV6 and SGIV reported here strengthen the results from previous and more recent studies, where these three isolates in addition to GIV and LBMV appear to deviate from the main cluster of ranavirus isolates closely related to EHNV and FV3 (Mao et al. 1997, Mao et al. 1999a, Hyatt et al. 2000, Qin et al. 2003, Song et al. 2004, Eaton et al. 2007). Recently, based on MCP gene sequences, Ohlemeyer et al. (2011) suggested that ranaviruses could be divided into three groups. The first group contains viruses originating from fish, reptiles and amphibians, such as EHNV, FV3, BIV, ECV/ESV, ATV and SSTIV, the second group consists of Santee Cooper ranaviruses LBMV and DFV/GV6, and the third comprises the grouper iridoviruses GIV and SGIV. Furthermore, it has been suggested based on a dot plot analysis of complete genome sequences that there are three genomic phenotypes among ranaviruses: FV3/TFV/STIV, ATV/EHNV and SGIV/GIV (Jancovich et al. 2011). Many of the known ranavirus isolates could be regarded as members of the same species, but separation of isolates has been favoured, as different ranaviruses have distinct host species and geographical ranges (Hyatt et al. 2000, Chinchar 2002, Chinchar et al. 2005, Williams et al. 2005). In most of the isolates studied here, except for GV6, DFV and SGIV, relatively little variation in MCP, DNApol, RNase III, RNR-α or -β sequences was observed (nt sequence identity >95 %). The greatest variation between the isolates was detected in NF-H1 sequences. NF-H1 gene homologues have been identified from the

genomes of several ranaviruses (Jancovich et al. 2003, Tan et al. 2004, Jancovich et al. 2010). In mammals, neurofilament triplet proteins are components of the neuronal cytoskeleton and are found in the neurons of both central and peripheral nervous systems (Scott et al. 1985, Carpenter & Ip 1996). Neurofilament proteins have also been identified in fish and amphibians (Dahl et al. 1986, Szaro & Gainer 1988, Mencarelli et al. 1991). Here, the variation between the NF-H1 sequences of different ranaviruses was mostly explained by the repetitive regions found in some of the isolates. The repeats in the NF-H1 sequences resulted in large gaps in the multiple sequence alignment, and the gap-forming repeats were excluded from the respective sequences in order to obtain sequences of similar length and to avoid overestimation of sequence differences. There are no definite rules in the alignment of repetitive units, and exclusion is one of the solutions used (Higgins 2003). Repetitive regions have been identified in the genomes of several ranaviruses, such as FV3, ATV, TFV, STIV and SGIV (He et al. 2002, Jancovich et al. 2003, Song et al. 2004, Tan et al. 2004, Huang et al. 2009). Genomes of other large DNA viruses, also including members of the family *Iridovirus*, contain repetitive sequences that are involved in viral genome replication and gene transcription (Tidona & Darai 1997, He et al. 2002, Delhon et al. 2003, Klupp et al. 2004, Williams et al. 2005).

The sequence analysis of the genes studied here clearly placed the previously less well known isolates, CodV, PPIV, REV 282/I02, Rmax and SERV, as close relatives to the other EHNV/FV3-like isolates, and as new members of the genus *Ranavirus*. The phylogenetic analyses revealed isolates of amphibian origin, REV 282/I02, FV3 and BIV, to be the closest relatives of PPIV, an isolate originating from a piscine host. CodV and Rmax, on the other hand, appeared to be closely related to EHNV. SERV, even though it was the most deviant isolate in the EHNV/FV3-like group of ranaviruses, showed the highest genetic relatedness to EHNV, ECV and ESV. It has been suggested based on sequence analyses of different ranavirus genomes that the ancestral ranavirus was a fish virus, and that several recent host shifts to amphibians, turtles and possibly back to fish lead to the speciation of viruses in their new host (Jancovich et al. 2010). It has also been hypothesized that ranaviruses may be able to cross numerous poikilothermic species barriers and can potentially cause disease in the new hosts. The genetic relatedness of PPIV and the amphibian ranavirus isolates is interesting considering that PPIV was originally isolated from symptomless pike-perch fingerlings (Tapiovaara et al. 1998). In challenge studies, PPIV caused no mortality but was able to persist in pike-perch and rainbow trout (Tapiovaara et al. 1998, Bang Jensen et al. 2011a), whereas mortality was observed in pike (Bang Jensen et al. 2009). The pathogenicity of PPIV to amphibians is unknown, but this isolate could possibly be an example of a ranavirus in the middle of a host-shift process, in which pike-perch acts as an intermediate host or a vector.

6.2 Detection and differentiation of ranaviruses

As new ranavirus isolates are identified and knowledge of the host range and pathogenicity of different ranaviruses accumulates, differentiation between isolates is becoming increasingly important. In the current OIE manual (OIE 2009, 2012), several methods are presented for the detection of EHNV and other ranaviruses. The antibody-based detection methods in the manual use polyclonal antibodies (PAbs), and it is stated that the antibodies cross-react with all known ranaviruses. PAb-based methods are therefore suitable for detecting ranaviruses as a group, but not for differentiating isolates. This was yet again demonstrated in study **II**, where complete cross-reaction was observed between all antisera and ranavirus isolates investigated. In recent studies by Gobbo et al. (2010) and Bang Jensen et al. (2009), positive immunofluorescence was observed with several ranavirus isolates when stained with PAb against ECV. Cinkova et al. (2010) reported similar results with a number of ranavirus isolates, except for GV6 and DFV, which could not be detected with PAb against ECV. Monoclonal antibodies (MAbs) have not been used as commonly in ranavirus detection. Reports have been published on MAbs–based detection methods for other iridoviruses, such as Lymphocystis disease virus and Red Sea bream iridovirus-like virus (Essbauer et al. 2004, Gibson-Kueh et al. 2004).

Molecular methods provide a means to differentiate the closely related ranaviruses from each other. Many of the published ranavirus detection methods are based on amplification followed by REA or sequencing analysis of the MCP gene (Mao et al. 1996, Mao et al. 1997, Hyatt et al. 2000, Marsh et al. 2002, Grizzle et al. 2003). The PCR-REA method published by Marsh et al. (2002) and recommended by the OIE differentiates Australian, European and American isolates. The PCR method and subsequent sequence analysis described by Hyatt et al.(2000), also included in the OIE manual, separates ranaviruses based on their differences in the MCP gene sequence. In this study, several PCR methods targeting different viral genes have been described. Differentiation of isolates was achieved either by REA or sequencing of the PCR products. Compared to the previous publications, additional, less well known isolates were included in the investigations. Altogether thirteen isolates were studied and detected using DNApol PCR (**I, II**), including the major group of EHNV/FV3-like isolates and the more deviant GV6, DFV and SGIV. In addition to conventional PCR, a ranavirus-specific and highly sensitive qPCR method based on the viral DNApol gene was developed for the detection and quantitation of ranaviruses (**III**), and is further discussed in section 6.4. DNApol is one of the best-studied proteins of large DNA viruses and has previously been used to determine viral phylogenetics (Goldberg et al. 2003, Getchell et al. 2007). Neither of the OIE manual methods was designed to detect SGIV, and as discussed in 6.1, the DFV and GV6 isolates studied by Hyatt et al. (2002) were different from those here. The PCR-REA methods described here differentiated nine of the eleven isolates studied, and DFV and GV6 as a group. Digestion of NF-H1 products with just one enzyme, *Hae*III, distinguished five known pathogenic isolates, EHNV, FV3, BIV, ECV and ESV, in addition to SERV. The methods presented in the OIE manual are not able to differentiate the closely related ECV and ESV from each other. Despite their high degree of genetic similarity, ECV and ESV

appear to differ in pathogenicity depending on the host species (Bang Jensen et al. 2009, Gobbo et al. 2010). Since both ECV and ESV are present in Europe, the ability to differentiate these isolates is important in the prevention of ranavirus infections. Ohlemeyer et al. (2011) recently published a PCR-REA method based on the MCP gene of ranaviruses that detected and differentiated several ranavirus isolates. However, grouper iridoviruses were not included in the study, and the method was also unable to distinguish between ECV and ESV.

Challenge studies with European fish species have demonstrated that several ranavirus isolates can infect the same fish species. Based on report by Gobbo et al. (2010), the black bullhead is susceptible to ECV and EHNV, but not to ESV, FV3, REV 282/I02, BIV or SERV. In addition to being susceptible to EHNV, ESV, PPIV and SERV, pike can act as a vector for ECV and FV3 (Bang Jensen et al. 2009). More information on the pathogenicity of ranaviruses, susceptible host species and possible vector species is needed to efficiently prevent the spread of these viruses. The ability to detect and differentiate closely related ranaviruses is an important factor in disease control.

6.3 Measurements of viral load in cell cultures and fish tissue

Four ranavirus isolates were propagated in EPC and BF-2 cells and the viral quantities were measured from seven time points during three days after inoculation. Additionally, viral load was determined from tissue samples of pike fry challenged with EHNV. Differences in viral quantities were observed between the two cell lines used. For EHNV and ECV, higher viral loads were detected in BF-2 cells, whereas for FV3 and DFV, the quantities were higher in EPC cells. Regarding ECV and FV3, similar results were obtained in a previous study that investigated propagation of ranaviruses in different conditions (Ariel et al. 2009b). In general, EHNV and FV3 reached the highest viral quantities of the isolates studied here, irrespective of the cell line used. Based on Ariel et al. (2009b), the incubation temperature and the cell lines used here could have been less than optimal for the propagation of ECV and DFV. The temperature also had an effect on the EHVN quantities in pike: elevated viral loads were detected earlier at 22 ºC than at 12 ºC. The viral quantities were in concordance with the mortalities observed in the challenge study. The highest mortality at 12 ºC was detected starting from day 7, whereas at 22 ºC the mortalities peaked 3-4 d. p.i. (Bang Jensen et al. 2009). Increasing temperature has been shown to reduce the incubation time of EHNV in redfin perch, rainbow trout and black bullhead (Whittington & Reddacliff 1995, Gobbo et al. 2010). Similarly, in a study in which white sturgeon were experimentally infected with white sturgeon iridovirus (WSIV), clinical signs were first observed at high temperatures (Watson et al. 1998).

Unlike conventional PCR methods, qPCR provides a means to study the quantity of viral particles in a given sample. Viral quantity is a useful indicator of the state of infection or disease outbreak. qPCR methods based on the MCP gene have previously been used to estimate viral loads of a single ranavirus isolate, such as LBMV (Goldberg et al. 2003,

Getchell et al. 2007). Here, a ranavirus-specific qPCR assay detecting ten different isolates has been described. The target of the assay was viral DNApol, a gene that had been successfully used in the broad-scale detection of ranaviruses in publication **I**. In order to obtain a viral load value, the quantity of virions was proportioned to the number of host cells using a qPCR targeting fish GK gene. Any possible differences in the measurements of viral quantities between samples due to the varying amounts of original template were normalized using the host DNA (GK gene) as an endogenous control. GK is a known single-copy gene in the genomes of several species such as humans, rats and common carp (Magnuson et al. 1989, Tanizawa et al. 1992, Panserat et al. 2000), and is therefore a suitable gene for estimating the number of host cells. GK has previously been used in the enumeration of koi carp (*Cyprinus carpio koi*) DNA while estimating the quantities of KHV in infected fish (Gilad et al. 2004).

Both DNApol and GK qPCR assays had a wide detection range regarding the target copy number. DNApol qPCR was ranavirus-specific: no amplification was observed when the DNA of RSIV or KHV was used as a template. GK qPCR detected host cell DNA from both EPC and BF-2 cells as well as from pike. Both methods generated highly reproducible results, with intra- and inter-assay CV-values well below 5 %, which is generally considered to be an acceptable level of variation (Islam et al. 2004, Abdul-Careem et al. 2006). In DNApol and GK qPCR described here, PCR products were detected with SYBR Green I, which binds to dsDNA independently of its sequence. Compared to the widely-used sequence-specific dual-labelled probes, SYBR Green chemistry is relatively affordable. Nevertheless, due to the non-specific binding of the detection dye, careful design of primers and assay optimization are necessary for specific results. Here, the qPCR primers were selected based on their specificity and amplification efficiency. Additionally, the specificity of amplification was controlled by using a hot-start DNA polymerase and by performing a melting curve analysis after each qPCR run. SYBR Green-based assays together with melting curve analysis have been widely used in the detection, quantitation and genotyping of different viruses, including iridoviruses (Payungporn et al. 2004, Pham et al. 2005, Ong et al. 2007, Gias et al. 2011, Wan et al. 2011). qPCR results are only indicative of the viral titre, since not all genomic material detected is necessarily from infective virions. Although qPCR can overestimate the number of virions, it is also probable that in a set of samples the number of target sequences is equal, resulting in an error of the same size in all samples (Islam et al. 2004).

6.4 Pathogenicity of ranaviruses to rainbow trout

The objective of the challenge trial was to compare the two Danish ranaviruses, CodV and Rmax, to other ranavirus isolates in terms of pathogenicity. Despite the fact that EHVN has repeatedly caused mortality in cultured rainbow trout in Australia (Langdon et al. 1988, Whittington et al. 1994), none of the ranavirus isolates used in the challenge trials (BIV, CodV, EHNV, ECV(Fr), ECV(It), ESV, FV3, PPIV, Rmax) were pathogenic to rainbow trout. Even though no signs of disease were observed, virus was isolated from a

few fish during the challenge, indicating the possibility of an established infection in those cases. The use of the original host species, such as cod or turbot for CodV and Rmax, may have resulted in a different level of pathogenicity to that observed here. CodV has previously been reported to cause disease symptoms in cod (Jensen & Larsen 1982). However, the results obtained here are in accordance with previous studies. Tapiovaara et al. (1998) observed no mortality in rainbow trout challenged with PPIV. It has also been shown that European stocks of rainbow trout and redfin perch are relatively well protected against EHNV in the low water temperature at which these species are normally cultured (Ariel & Bang Jensen 2009). The water temperature used here (15 ºC) may have impacted on the infectivity of the isolates. Temperature reportedly affects the immune response in fish (Hardie et al. 1994, Le Morvan et al. 1998, Watson et al. 1998) as well as the replication of ranaviruses (Chinchar 2002). ESV caused high mortality in sheatfish (Wels catfish) cultured in warm water in Germany in 1988 (Ahne et al. 1989). Challenge trials with ESV in sheatfish cultured in 24 ºC resulted with 100% mortality after bath exposure or co-habitation with infected fish (Ahne et al. 1990). According to Whittington and Reddacliff (1995), rainbow trout were susceptible to EHNV by i.p. inoculation at temperatures ranging from 8 to 21 ºC and that the incubation time increased with decreasing temperature. Similarly, mortality was observed earlier in black bullhead bath exposed to EHNV or ECV at 25ºC compared to fish held at 15 ºC (Gobbo et al. 2010). Additionally, virus was not isolated from all surviving black bullheads maintained at 25 ºC, and it was speculated that the host may have succeeded in eliminating the virus, and that the carrier status of the host may be dependent on water temperature. Viral infection occurring at low temperatures could result in chronic conditions, as was observed with white sturgeon (*Acipenser transmontanus*) infected with white sturgeon iridovirus (WSIV) (Watson et al. 1998). In this study it is possible that no infection was established at 15 ºC, or that only subclinical infection was induced in the majority of the fish. Persistent infection with EHNV in i.p.-inoculated rainbow trout was also observed by Whittington and Reddacliff (1995) in subclinical fish 63 days after exposure. Additionally, virus could be isolated up to 24 days post-challenge from symptomless rainbow trout fry challenged with PPIV (Tapiovaara et al. 1998). The results obtained here strengthen the view that rainbow trout could act as a vector for ranaviruses. Additionally, the water temperature seems to be an important factor in the pathogenesis of ranaviruses, and should be taken into account with European fish cultured in warm water and when transferring fish from colder to warmer conditions.

The infective dose applied and the characteristics of the host animal have been reported to affect viral pathogenesis. Experimental infection studies with tiger salamander larvae and northern leopard frog tadpoles demonstrated that increasing doses of FV3 or ATV increased mortality, even though with tiger salamander this trend was not observed when the animal densities were higher (Brunner et al. 2005, Echaubard et al. 2010). The host physiological phenotype is one factor that has been shown to explain why hosts vary in their potential to function as pathogen reservoirs (Cronin et al. 2010). In a pathogenicity study on ATV, the life history traits and genetic background of the host were found to affect the virulence (Brunner et al. 2005). The combination of the experimental conditions,

including the viral doses and the relatively low water temperature used, could have prevented the onset of the disease in the present study. In addition, the genetic properties of the rainbow trout strain used here may have enabled the fish to resist the infection.

Both the OIE and EU currently only list one ranavirus, EHNV, as a notifiable pathogen for fish. Of the European fish species, EHNV is able to infect pike, pike-perch and black bullhead in challenge conditions (Bang Jensen et al. 2009, Gobbo et al. 2010, Bang Jensen et al. 2011a), whereas redfin perch and rainbow trout appear less susceptible (Ariel & Bang Jensen 2009, **II**). So far, EHNV has only been isolated in Australia (Langdon et al. 1986, Langdon et al. 1988). In Europe, on the other hand, ranavirus isolates pathogenic to fish, including ECV and ESV, have been characterised (Ahne et al. 1989, Pozet et al. 1992). In addition, potentially pathogenic isolates found in Europe, such as PPIV and SERV, have demonstrated infectivity in challenge studies (Bang Jensen et al. 2009). As shown in publications **I** and **II**, as well as in other studies (Hyatt et al. 2000, Ariel et al. 2009a, Ohlemeyer et al. 2011), all these isolates have a high degree of genetic similarity with EHNV. Therefore, it has been suggested that the genetic properties in addition to the pathogenicity of different isolates should be taken into consideration when deciding on the listing and surveillance of these viruses (Bang Jensen et al. 2011a).

6.5 Immune response to ranavirus infection

In study **IV**, the immune response to ranavirus infection in a piscine host was investigated using a fish epithelial cell line. qPCR assays were developed to measure and characterise the expression of selected immune genes in ranavirus-infected epithelial cells. In vertebrates, epithelial tissues form a barrier between the body and the environment and play an essential role in host defence and regulation of the immune response. In mammals, epithelial cells respond to infection or injury by secreting cytokines and chemokines (Stadnyk 1994, Laan et al. 1999). In addition to acting as primary innate immune effector cells, epithelial cells regulate adaptive immune responses at the level of leukocytes (Schleimer et al. 2007). The epithelial cells used here were EPC cells, a cell line widely used in diagnostic and research laboratories for viral fish diseases. The sequencing results obtained here for the COx1 and immune genes of EPC cells were in accordance with the findings published by Winton et al. (2010) and further confirmed that the current lineages of EPC cells originate from fathead minnow instead of common carp.

All four ranaviruses induced an immune response in EPC cells, despite the fact that DFV deviates genetically from EHNV, ECV and FV3 (**I**, Mao et al. 1997, Hyatt et al. 2000, Ohlemeyer et al. 2011). The expression profiles of the immune genes differed between the viral treatment groups, however. FV3 and EHNV induced a strong pro-inflammatory response by up-regulating both TNF-α and IL-1β. ECV and DFV, on the other hand, only induced a statistically significant increase in TNF-α expression. In addition, the relative viral load values of FV3, EHNV and DFV correlated with the IL-1β expression, indicating that the increasing amount of virions enhanced the pro-inflammatory cytokine production

in EPC cells. In rainbow trout, viral infection has been shown to induce a significant increase in TNF-α and IL-1β expression (Purcell et al. 2004). Similar results have been obtained with FV3-infected *Xenopus* (Morales et al. 2010), and SGIV reportedly induced the up-regulation of TNF-α in grouper (*Epinephelus* sp.) spleen cells (Huang et al. 2011a).

Since both TNF-α and IL-1β have been associated with the induction of apoptosis (Boehm et al. 1997, Cooper et al. 2001, dos Santos et al. 2008), the up-regulation of TNF-α and IL-1β was consistent with the apoptotic changes observed in this study. It has been demonstrated in previous studies that FV3 induces apoptosis in fish, amphibian and mammalian cells (Chinchar et al. 2003, Morales et al. 2010). Furthermore, Huang et al. (2011b) found that SGIV caused apoptosis or non-apoptotic programmed cell death depending on the cell type. Other ranaviruses, such as SSTIV and GIV, have also been reported to induce apoptosis (Chiou et al. 2009, Huang et al. 2011c). Here, apoptotic changes were observed in all ranavirus treatment groups. Apoptosis was investigated using dyes that bind to all dsDNA, including host and viral DNA. Apoptotic cells were detected by their distinct features, such as condensed chromatin and fragmented nuclei. An apoptosis-specific method using Annexin V, a protein that binds to phosphatidylserine present in the surface of apoptotic cells (Koopman et al. 1994), could have detected cells at the early stages of apoptosis, before the major morphological changes. Additionally, it would have been beneficial to include a positive control group treated with an apoptosis inducer, such as camptothecin or actinomycin D, to the study.

Up-regulation of β2M was induced relatively quickly after inoculation by all four ranaviruses. The increase detected in β2M expression suggests that ranaviruses induce an antigen-specific immune response leading to the activation of cytotoxic lymphocytes in a piscine host. Several studies have demonstrated that viral infection induces increased β2M expression in fish. In rainbow trout infected with infectious hematopoietic necrosis virus (IHNV), β2M transcription was up-regulated in the spleen and intestine (Hansen & La Patra 2002). Increased β2M expression was also observed in the head kidney of Atlantic salmon (*Salmo salar*) infected with infectious salmon anaemia virus (ISAV) (LeBlanc et al. 2010).

IL-10 is a regulatory cytokine mainly produced by the cells of the immune system (Saraiva & O'Garra 2010). However, human epithelial cells reportedly regulate immune reponse through the expression of IL-10 (Bonfield et al. 1995, Jarry et al. 2008). Interestingly, IL-10 expression was detected here in EPC cells and ranavirus infection seemed to affect the expression levels. IL-10 was up-regulated in the ranavirus-infected EPC cells at the early time points of this study. Later on, the IL-10 expression levels decreased relative to the negative control, while IL-1β and TNF-α expression increased. The expression of TGF-β, another cytokine with regulatory functions, was transiently induced in EPC cells infected with ECV and DFV. TGF-β has been shown to enhance viral replication in humans (Li et al. 1998, McCann & Imani 2007). The modest increase in TGF-β expression detected in this study could be related to the replication strategy of ECV and DFV.

Of the bacterial stimulants used in this study, both LPS and CpG-ODN induced a pro-inflammatory response in EPC cells. LPS induced the most notable increase in TNF-α and IL-1β expression, whereas CpG-ODN elicited a more modest up-regulation of β2M, IL-1β and TNF-α. The results obtained are in accordance with previous studies on carp and rainbow trout head kidney phagocytes (Zou et al. 2000, Engelsma et al. 2001, Stolte et al. 2008, Teles et al. 2011), in which it was shown that LPS-stimulation evoked a rise in the expression of pro-inflammatory cytokines. CpG-ODN stimulation reportedly induced the expression of IL-1β in rainbow trout (Jørgensen et al. 2001) and of TNF-α, IL-1β and MHC class I surface molecules in mammals (Decker et al. 2000, Krieg 2002). Poly I:C, a synthetic viral-like dsRNA, is generally used to mimic viral infections (Fortier et al. 2004, Purcell et al. 2004). For EPC cells, poly I:C appeared to be a strong stimulant; it induced up-regulation in all the immune genes studied. In previous studies, poly I:C stimulation reportedly elicited β2M and TGF-β expression in fish (Zhan & Jimmy 2000, Purcell et al. 2004, Zheng et al. 2006).

As discussed earlier, the species, genetic background and the life history stage of the host as well as the ambient conditions may have an effect on the pathogenesis of ranavirus infection. EHNV has been shown to be pathogenic to several Australian and European fish species (Langdon et al. 1988, Langdon 1989, Bang Jensen et al. 2009, Gobbo et al. 2010, Bang Jensen et al. 2011a). A known pathogen for amphibians, FV3, caused no mortality in either pike or pike-perch, but the virus could be re-isolated from the fish after a bath-challenge, indicating that both fish species could act as a vector for the virus (Bang Jensen et al. 2009, Bang Jensen et al. 2011a). ECV is pathogenic to black bullhead and has been shown to persist in pike and pike-perch without causing significant mortality (Pozet et al. 1992, Bang Jensen et al. 2009, Gobbo et al. 2010, Bang Jensen et al. 2011a). On the other hand, DFV along with EHNV, FV3 and ECV neither persisted nor caused mortality in carp or goldfish (Bang Jensen et al. 2011b). The immune gene expression results obtained in this study provide preliminary evidence that fathead minnow epithelial cells produce a measurable immune response against ranaviruses. Furthermore, different ranavirus isolates appear to induce distinct response patterns. More studies with primary cells and ultimately with living piscine hosts are needed to confirm these results and to further elucidate the immune processes activated by different ranaviruses in different host species.

7 Conclusions

1. The viral gene sequences analysed in this study further support the view that the genus *Ranavirus* is genetically divided. The main group of ranaviruses includes the numerous EHVN/FV3-like isolates, whereas the Santee-Cooper ranaviruses and the grouper iridoviruses form separate groups. The less well characterised isolates CodV, PPIV, Rmax, REV 282/I02 and SERV belong to the main group, in which the nucleotide sequence identity among different ranaviruses is relatively high.

2. Both the OIE and EU list one ranavirus, EHNV, as a notifiable fish pathogen, whereas for amphibians all ranavirus infections are listed by the OIE. To meet the diagnostic needs, new methods for identification of a wide variety of ranaviruses were developed in this study. EHNV as well as several closely related and more deviant ranavirus isolates were detected and differentiated with PCR and subsequent restriction enzyme analysis of the viral DNApol and NF-H1 genes. The quantitative DNApol PCR was proven to be a specific and sensitive method that could be used in high-throughput screening and quantitation of ranaviruses as well as in studies of ranavirus pathogenesis.

3. In contrast to the reports of EHNV outbreaks in Australia, the European strain of rainbow trout was not susceptible to EHNV, BIV, CodV, ECV, ESV, FV3, Rmax or SERV in the challenge conditions used in this study. However, succesfull viral isolation from few of the fish in the challenge experiment indicates that rainbow trout could act as a vector for ranaviruses. In addition to the genetic properties of the host, the relatively low water temperature may have affected the infectivity of the ranaviruses studied.

4. Ranavirus infection up-regulated pro-inflammatory cytokines, induced changes in the regulatory cytokine expression, activated the MHC class I antigen presentation pathway and elicited apoptosis in fish epithelial cells. The immune response induced varied between the ranaviruses, which could be associated with the differing host specificities of the isolates studied.

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